University of Alberta

Relative mRNA expression of ghrelin, leptin and stearoyl-CoA desaturase in Holstein bull calves injected with ACTH

by

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Abstract

Impact of acute stress on key factors involved in feed regulation and energy storage could provide insight into feed efficiency and growth of calves. The objectives of this study were to examine ghrelin mRNA expression in calf tissues and determine the influence of adrenocorticotrophin hormone (ACTH) on ghrelin, leptin and stearoyl-CoA desaturase (SCD) mRNA expression. Five-month old Holstein bull calves (n=12; 3/group) were euthanized at 15 (AH15), 50 (AH50) or 100 (AH100) minutes following injection of 0.56 IU/kg ACTH or immediately following saline injection (SALINE). Twenty-one tissue types were collected for determination of relative mRNA expression. Plasma cortisol was significantly increased in AH100 calves relative to SALINE calves. Relative to pooled data from saline calves (CONTROL), relative leptin mRNA expression increased in the small intestine of AH50 calves, relative liver SCD mRNA expression decreased in AH15 and AH50 calves, and SCD mRNA expression increased in splenic samples from AH100 calves.

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List of Abbreviations

3βHSD	3β-hydroxysteroid dehydrogenase
ACAT	Acyl-CoA cholesterol acyltransferase
ACTH	Adrenocorticotrophin hormone
ADG	Average daily gain
AGRP	Agouti-related protein
α-MSH	α-Melanocyte stimulating hormone
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine- regulated transcript
CCK	Cholecystokinin
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CRS-1	cAMP-responsive sequence 1
CRS-2	cAMP-responsive sequence 2
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DRU	Dairy Research Unit
E	Efficiency
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GHS	Growth hormone secretagogue
GHS-R	Growth hormone secretagogue receptor
GLUT-2	Glucose transporter-2
G_s	Stimulatory G-protein
HPA	Hypothtlamic-pituitary-adrenal
HSL	Hormone sensitive lipase
IRS-1	Insulin receptor substrate-1
Jak2	Tyrosine kinase Janus kinase-2
MAG	Monoacylglycerol
MAPK	Mitogen-activated protein kinase
MGB	Minor groove binding
mRNA	Messenger RNA
MUFA	Mono-unsaturated fatty acid
NEFA	Non-esterified fatty acids
NPY	Neuropeptide Y
Ob-R	Leptin receptor
PFK-2	Phosphofructokinase-2
PKA	Protein kinase A

PKC	Protein kinase C
POMC	Pro-opiomelanocortin
SCD	Stearoyl-CoA desaturase
SFA	Saturated fatty acid
SGLT-1	Sodium glucose transporter-1
StAR	Steroidogenic acute regulatory protein
STAT3	Singal transducer and activator of transcription-3
TAG	Triacylglycerol
TMR	Total mixed ration
WAT	White adipose tissue

1. LITERATURE REVIEW

Introduction

Feed intake is a primary concern in the dairy industry because of its importance in growth, lactation, efficiency of milk production, feed efficiency and rumen health. Nutrient intake is central to growth, lactation and reproductive efficiency. Environment, physiology and genetics all affect feed intake; however, these factors also interact with each other. The production of adrenocorticotrophin hormone (ACTH) in response to environmental stressors has received limited attention in ruminant research. However, environmental stress could prove to be a significant regulator of hormones related to feed intake, such as ghrelin and leptin, during growth and development in young ruminants.

In the steroidogenic pathway, ACTH is secreted by the anterior pituitary in response to stress stimuli and it increases concentrations of free cholesterol in the adrenal cortex. This elevation occurs via increased hydrolysis of cholesteryl esters, increased endocytosis of free cholesterol and increased *de novo* synthesis of free cholesterol. Free cholesterol is then imported to the inner mitochondrial membrane via the steroidogenic acute regulatory protein (StAR) where it undergoes a series of transformations through the action of numerous enzymes, including cytochrome P450 enzymes, to yield the glucocorticoids corticosterone and cortisol. Cortisol is the major glucocorticoid associated with stress in ruminants, and induces a whole body response, affecting feed intake, metabolism, growth and immune function (Squires, 2003).

Glucose and fatty acid metabolism are affected by cortisol. Increased

circulating cortisol results in inhibited gut glucose uptake and glucose utilization by peripheral tissues. Fatty acids are rapidly mobilized for energy usage, creating shifts in expression and/or abundance of enzymes involved in triacylglyceride processing, such as stearoyl-CoA desaturase.

The effects of increased cortisol secretion on feed intake is mediated through hormones that regulate feed intake. Leptin and ghrelin are major hormones involved in the suppression and stimulation of feed intake, respectively. Suppression of feed intake as a result of leptin secretion from white adipose tissue was originally thought to be leptin's primary function; however, the recent discovery of leptin messenger RNA (mRNA) expression in other tissues such as the stomach and small intestine has led to the suggestion that leptin secretion from other tissues is involved in carbohydrate absorption across the brush border of the small intestine (Bado et al., 1998; Yonekura et al., 2002).

Ghrelin is the first known natural ligand for growth hormone secretagogue receptor (GHS-R) and induces growth hormone (GH) secretion from the anterior pituitary (Date et al., 2000a; Date et al., 2000b). Ghrelin also stimulates feed intake via neuropepetide Y (NPY) and Agouti-related protein (AGRP) neurons in the hypothalamic arcuate nucleus (Korbonits and Grossman, 2004). Ghrelin is a potent orexigenic hormone secreted primarily by oxyntic glands in the monogastric stomach; however, there has also been evidence that it is secreted by other tissues such as the brain, kidney and lung (Ariyasu et al., 2001; Kojima et al., 1999; Mori et al., 2000). Extrapolation of expression and secretion patterns in ruminants based on monogastric findings must be done with caution, as differences in ghrelin potency

have already been identified between monogastrics and ruminants (Hashizume et al., 2005; Itoh et al., 2005). To date, ghrelin mRNA expression has only been studied in the gastrointestinal tract of ruminant animals (Hayashida et al., 2001). A recent suggestion that ghrelin concentration may be more potently regulated by macronutrient metabolism rather than endocrine stimulus (Erdmann et al., 2003; Sanchez et al., 2004) has triggered investigation into the direct effects of glucose and insulin on ghrelin abundance and circulation (Caixas et al., 2002; Saad et al., 2002; Williams et al., 2003a). Furthermore, relationships between ACTH, cortisol and ghrelin have been observed in monogastrics where ghrelin injection increased the release of ACTH and cortisol in humans and rodents (Broglio et al., 2004b; Schmid et al., 2005). However, the effects of these stress hormones, as well as the influence of glucose and insulin, on ghrelin expression in ruminants have yet to be determined.

1. Feed Intake

1.1. Environment, physiology and genetics

Intake of macronutrients, such as proteins, carbohydrates and fatty acids is required to maintain biological function, including energy production, in the body. The body also has the ability to retain macronutrients in several forms, to be stored and utilized when the body is deficient in energy. Such a deficient condition includes fasting, when there is no macronutrient intake and the body relies on its energy stores to provide fuel for energy demands. Other conditions also exist where an animal is consuming feed but intake is insufficient to meet energy demands, such as

during early lactation in dairy cattle. Energy demands are influenced by numerous factors, including growth, maintenance and lactation. Feed can provide the body with forms of macronutrients that would be otherwise unavailable due to the mammalian's biological inability to produce those forms. An example of this is dietary fat, which is reflected by the differences in the *de novo* synthesis capabilities of animals and plants. Plants have the ability to desaturate fatty acids at different carbons than animals (plants desaturate at carbons 9, 12 and 15 while animals desaturate carbons 5, 6 and 9) (Vance and Vance, 2002), and hence it is necessary for animals to acquire plant-derived fatty acids via dietary means and to have the ability to manipulate these dietary fatty acids. Stimulation and suppression of feed intake are influenced by many hormones and peptides secreted within the body (Ingvartsen and Andersen, 2000; Sahu, 2004). *Table 1.1.* provides a general list of these regulators.

In addition to these complex physiological and dietary contributions, other factors such as environment, physiological state and genetics also affect nutrient requirements, which then influence intake. Stress is an example of an environmental impact on energy demands and the body responds by increasing production of glucose and fatty acids. Other factors such as genetic basal expression and circulatory threshold concentrations of hormones related to feed intake and behavioural responses to stress also affect energy demands (Jones et al., 1992; Vonborell, 1995). There is considerable variation between individuals in the peak and amplitude of endocrinological responses to a stress stimuli.

1.2. Impact of stress on feed intake

Many rodent studies have utilized various stressor stimuli to examine hormonal responses to both acute and chronic stress. Alterations in feed intake as a result of a stress stimuli are influenced by the type of stress and the duration of the stressful period, along with other contributing factors (Charmandari et al., 2005). In accordance with cortisol's differential response to acute or chronic stress, regulation of neuropeptide Y (NPY) is dependent on the stress type experienced. Neuropeptide Y is a potent stimulator of feed intake and is secreted from the hypothalamus to elicit an orexigenic response. Acute stress administered in the form of random foot shocks demonstrated an increase in NPY expression in rats (Kas et al., 2005) while chronic stress induction via a series of different stressful events resulted in a decrease in hypothalamic NPY expression in rats (Kim et al., 2003).

Typical environmental stress stimuli, such as handling, isolation or milking can increase blood cortisol in cattle (Friend, 1991; Grandin, 1993; Munksgaard and Simonsen, 1996; Negrao et al., 2004; Ting et al., 2004). Even within their home environment, relatively minor changes such as different feed, a novel object, or a new worker can induce stress stereotypes in cattle (Herskin et al., 2004). Social rank within the herd is not necessarily correlated with cortisol concentrations, as even between individual non-agonistic animals there is a large variation in basal cortisol concentrations (Mulleder et al., 2003). Stress challenges in both calves and mature cattle that mimic typical physiological adrenocorticotrophin hormone (ACTH) appear relatively consistent (Negrao et al., 2004; Verkerk and Macmillan, 1997). It is generally agreed that acute stress stimulates appetite while chronic stress

suppresses appetite, while nutrient uptake and utilization are also depressed (Ingvartsen, 1994; Sapolsky et al., 2000). These circumstances prove unfavourable for producers, as decreased appetite negatively affects growth and decreased nutrient uptake and utilization decreases feed efficiency and production parameters.

Corticotrophin-releasing hormone (CRH) is thought to suppress feed intake and is secreted primarily by the paraventricular nucleus (Ingvartsen and Boisclair, 2001) to stimulate ACTH secretion. Gene expression and secretion of CRH is influenced by other hormones such as insulin and leptin, and is directly affected by feed intake (Chaillou et al., 2002). Stress induces direct biological shifts in rate and amplitude of metabolic and melanocortin pathways while it also induces indirect changes through the regulation of feed intake. Dietary intake of macronutrients mediates enzymatic and hormonal regulation. For example, specific fatty acids that tend to be higher in unsaturated double bonds regulate gene transcription of enzymes required for fatty acid storage and mobilization (Daniel et al., 2004; Giacometti et al., 2005; Jump and Clarke, 1999; Nakamura and Nara, 2002).

Though variable and contradictory findings have been reported, it is generally noted that cortisol does follow a circadian rhythm or is suppressed post-prandially in some species (Purnell et al., 2003). Though no diurnal pattern of cortisol secretion was originally observed in ruminants, pre-ruminant dairy calves have been shown to have sharp post-prandial decreases in blood cortisol, along with a significant increase during the dark period (Gardygodillot et al., 1989). However, these pre-weaned calves were fed at specific time points, and hence cortisol suppression could be due to the fact that nutrients were rapidly absorbed in

the abomasum in contrast to the slower digestion of ruminant animals. Although this study does demonstrate that feed intake does impact cortisol secretion, young cattle with developing rumens or mature cattle may not display a diurnal pattern of cortisol secretion, particularly if fed an *ad libitum* diet.

2. The Steroidogenic Pathway

2.1. Introduction to the steroidogenic pathway

The mammalian stress response is complex and involves numerous physiological and behavioral components. In addition to receiving and processing information via the central nervous system (CNS), the sympathetic nervous system is responsible for the involuntary physiological responses resulting from stress. In response to an environmental stressor, an immediate short-term response is triggered by the sympathetic nervous system to stimulate adrenaline secretion from the adrenal medulla, while the hypothalamic-pituitary-adrenal (HPA) axis is stimulated as a long-term response to stress (Charmandari et al., 2005). The HPA axis is responsible for fluctuations in CRH, melanocortin peptides and cortisol. Adrenocorticotrophin hormone (ACTH) is one of the melanocortin peptides derived from post-translational modification of the melanocortin peptide pro-opiomelanocortin (POMC) (Irani and Haskell-Luevano, 2005). Adrenocorticotrophin hormone is secreted from the anterior pituitary and regulates secretion of cortisol and corticosterone, both of which are glucocorticoids, from the zona fasciculata (or middle) of the adrenal cortex (Conley and Bird, 1997).

In the process of adrenal steroid biosynthesis, free cholesterol is taken up by

the adrenal cortex, and cytosolic cholesteryl esters are hydrolyzed from lipid droplets to form free cholesterol. *De novo* synthesized free cholesterol also contributes to the free cholesterol pool, and the steroidogenic acute regulatory protein (StAR) imports free cholesterol to the inner membrane of mitochondria located in the zona glomerulosa of the adrenal cortex. As seen in *Figure 1.1.*, cholesterol is then metabolized for the purpose of steroid biosynthesis within the mitochondria, undergoing a series of transformations in the mitochondria and endoplasmic reticulum of the adrenal cortex, yielding cortisol (Conley and Bird, 1997; Li et al., 2002). The cyclic adenosine monophosphate (cAMP) signaling pathway is activated by ACTH stimulation, inducing stimulation and in some circumstances increased abundance of the enzymes involved in the cortisol biosynthetic pathway. Stimulated enzymes include those from the cytochrome P450 superfamily (*P450c17*, *P450scc* and *P450c21)* and 3β-hydroxysteroid dehydrogenase (3βHSD) (Vance and Vance, 2002).

2.2. Cortisol stimulation of gluconeogenesis and lipolysis

Catecholamines, such as epinephrine and norepinephrine, constitute the primary "fight or flight" response by rapidly redirecting blood flow, increasing lung ventilation and rapidly mobilizing energy stores (Squires, 2003). Cortisol is also a hormone involved minutes after this primary response, stimulating liver gluconeogenesis, fatty acid mobilization, and gastric secretion, while decreasing the rate of cellular glucose transport and uptake of glucose by gastrointestinal tissues (Squires, 2003). Increased gluconeogenesis and decreased glucose utilization by

cells significantly increases blood glucose concentrations. Under feed-induced hyperglycemia, plasma insulin concentrations typically increase, stimulating the conversion of fructose-6-phosphate to fructose-2,6-bisphosphate via phosphofructokinase-2 (PFK-2) which in turn decreases blood glucose concentrations. Cortisol inhibits insulinemic effects on glucose and lipid metabolism while cortisol acts synergistically with insulin to increase glycogen deposition (Sapolsky et al., 2000). Whether increased insulin results from increased blood glucose or whether it is directly affected by cortisol remains unclear. Elucidation of the specific regulatory actions of cortisol have been attempted, with particular focus on determination of the interactions between glucose, cortisol and insulin.

Fatty acids are mobilized from adipose stores during the cortisol response (Sapolsky et al., 2000; Squires, 2003). Increased lipolysis is not induced directly by cortisol, but rather through decreased re-esterification of fatty acids (Leung and Munck, 1975). Catecholamines promote lipolysis by binding to β-adrenoreceptors. These β-adrenoreceptors activate adenylate cyclase via the stimulatory G-protein (G_s), adenylate cyclase stimulates conversion of adenosine triphosphate (ATP) to cAMP. Protein kinase A (PKA) is then bound to cAMP via cAMP phosphodiesterase and the cAMP-PKA complex then phosphorylates hormone sensitive lipase (HSL) to its active form. Hormone-sensitive lipase consequently hydrolyzes triacylglycerol (TAG) and diacylglycerol (DAG) to form DAG and monoacylglycerol (MAG), respectively, releasing free fatty acids for energy usage (Vance and Vance, 2002), as outlined in *Figure 1.2.* In response to cortisol, there is also increased expression of β-adrenoreceptors through glucocorticoid response elements that are upstream

from the β-adrenoreceptor gene. Cortisol also increases G_s mRNA expression and protein activity (Vance and Vance, 2002).

2.3. Cortisol secretion in cattle

Plasma cortisol concentrations in young dairy bulls is approximately 4 to 6 ng/mL when the calves are 5 weeks of age, and gradually increases to concentrations of approximately 8 to 15 ng/mL in mature dairy cattle (Gardygodillot et al., 1989; Negrao et al., 2004; Verkerk et al., 1994). Injection of 0.6 IU/kg of ACTH into 3-month-old Holstein calves resulted in plasma and salivary cortisol peaks approximately 40 minutes post injection (Negrao et al., 2004). Lactating multiparous cows have a similar response to ACTH administration; however, injection of multiparous Holstein-Friesian cows with approximately 0.68 IU/kg of ACTH yielded a higher cortisol response and peaked at 50 minutes (Verkerk et al., 1994). Cortisol peaks were 67 ± 4 ng/mL at 50 minutes post injection for cows, versus approximately 42 ng/mL at 40 minutes in calves (Negrao et al., 2004; Verkerk and Macmillan, 1997; Verkerk et al., 1994). Cortisol response of bulls to ACTH administration is less than that observed in cows, and cortisol response further decreases as the age of bulls increases, with peak cortisol decreasing from 52 ± 4 ng/mL at 6 months of age to 35 ± 3 ng/mL at 10 months of age. Steers also demonstrate an increased cortisol response to ACTH as compared to bulls as age increases, despite no significant association between testosterone and cortisol response being observed (Verkerk and Macmillan, 1997).

2.4. Involvement of the cytochrome P450 family in cortisol synthesis

Cortisol biosynthesis is regulated not only by StAR, but also by members of the cytochrome P450 enzyme superfamily. As displayed in *Figure 1.1.*, P450scc, P450c17, P450c21, P450c11 and P450aldo convert cholesterol to cortisol and aldosterone after influx of free cholesterol to the inner mitochondrial membrane via StAR. Regulation of these enzymes occurs predominantly through transcriptional regulation of the CYP genes, which encode for the P450 enzymes. The bovine CYP17 gene, which encodes for P450c17, was first characterized in 1989 (Bhasker et al., 1989). Expression of CYP17 has been identified in the adrenal, testis, ovary, stomach, placenta and brain (Bhasker et al., 1989; Weng et al., 2005; Yamazaki et al., 1998). The P450c17 enzyme is involved in androgen production as well as cortisol production, hence its expression in gonadal tissue. Structural characterization of the bovine CYP17 gene has also permitted determination of regulatory sites of the gene. The cAMP-responsive sequences have been identified in the promoter region of the CYP17 sequence (Lund et al., 1997).

There are two regions in which cAMP can regulate CYP17. These two regions are termed cAMP responsive sequence 1 (CRS1), -243 to -225 basepairs from the transcription start site, and cAMP responsive sequence 2 (CRS2), -80 to -40 basepairs relative to the transcription start site (Lund et al., 1990; Zanger et al., 1991). Though both of these regions are regulated by cAMP, elucidation of how each region is specifically regulated has demonstrated that both protein kinase A (PKA) and protein kinase C (PKC) signal transduction pathways are stimulated (Bakke and Lund, 1992; Lund et al., 1997).

Two pathways of steroid synthesis occur in the majority of mammals (Δ4 and Δ5 pathways); however, primates, humans, cattle and sheep utilize only the Δ5 steroid pathway (Conley and Bird, 1997; Yamazaki et al., 1998). In the Δ4 pathway, the Δ5 bond of pregnenolone is first isomerized by 3βHSD. Ketones subsequently bind to the third carbon, forming Δ4 progesterone. In the case of the Δ5 pathway, the Δ5 bond remains intact because pregnenolone is first manipulated by P450c17 (Conley and Bird, 1997). Treatment of bovine adrenocortical cells with ACTH results in increased conversion of pregnenolone to 17αhydroxypregnenolone, insinuating an increase in P450c17α activity and consequently a significant increase in cortisol production (Zuber et al., 1985). Fluctuations in messenger RNA (mRNA) expression and enzymatic protein yields have shown a strong positive correlation between CYP17 mRNA and P450c17α protein levels (LeHoux et al., 1992). This strong positive correlation, along with the enzyme's significant role in bovine cortisol production, renders P450c17α an ideal enzymatic candidate for indication of ACTH stimulation of cortisol biosynthesis.

3. Ghrelin

3.1. Ghrelin expression

Ghrelin expression was originally found in the acid-secreting cells of the rat stomach, known as X/A like cells or ghrelin cells, which are contained within the oxyntic mucosae (Date et al., 2000a). Though the main site of ghrelin mRNA expression is still thought to be in the stomach, ghrelin expression has also been discovered in numerous other tissues, including the heart, lung, lymphocyte,

pancreas, small intestine, kidney, pituitary, placenta, testis, ovary, arcuate nucleus, hypothalamus and adrenal cortex (Hayashida et al., 2002; Kojima et al., 1999; Mori et al., 2000; Tortorella et al., 2003). However, data examining expression sites in ruminants is limited, as only the ruminant stomach and small intestine have been examined as potential ghrelin secreting tissues (Hayashida et al., 2001).

A recent publication identified a conserved region within the ghrelin sequence that is thought to be cleaved from the preprotein, creating the Obestatin polypeptide. Obestatin suppresses feed intake and body weight gain, as well as gastric emptying and contractile activity in the jejunum using a distinct receptor (Zhang et al., 2005). Confirmation of this peptide has not yet been made; however, it now provides the interesting possibility that stimulation of the ghrelin gene will induce secretion of the counter-regulatory hormones ghrelin and obestatin.

3.2. Age differences influence ghrelin

Plasma ghrelin increases with age in both ruminants and monogastrics, as demonstrated by comparing plasma ghrelin in mature cows versus 3 month old calves (Miura et al., 2004). However, ghrelin mRNA expression has been reported to be similar between both young and mature cattle (Wertz et al., 2004). Developmentally, ghrelin mRNA expression has been observed to be present in rat fetal pancreatic ε cells, thyroid and lung (Chanoine, 2005; Chanoine and Wong, 2004; Wierup et al., 2004). It remains unclear at what stage of development other tissues, primarily the stomach of monogastrics, become the main sites of ghrelin secretion (Chanoine, 2005; Chanoine and Wong, 2004). A shift in primary

expression site could indicate a shift in ghrelin's role in carbohydrate metabolism or a shift in glucose, insulin and glucagon influence on ghrelin production.

3.3. Ghrelin as an endogenous ligand

Growth hormone secretagogue (GHS) is a synthetic compound that has been identified as a key stimulator of growth hormone (GH) release through binding to GHS receptors (GHS-R) located in the anterior pituitary and to a lesser extent in the hypothalamus, stomach, heart, lung, intestine, adrenal cortex, adipose tissue, immune system and pancreas (Lai et al., 2005; Mori et al., 2000). The presence of GHS-R in pancreatic cells remains under scientific debate (Kageyama et al., 2005; Mondal et al., 2005; Papotti et al., 2000). Growth hormone secretagogue receptors are seven-transmembrane G protein-coupled receptors (Lai et al., 2005). Ghrelin was isolated and identified as a natural ligand of GHS-R (Kojima et al., 1999). Two forms of the 28 amino acid ghrelin have been isolated, the 'inactive' des-acyl ghrelin and the 'active' acyl ghrelin, on which the Ser3 has a *n-octanoyl* group attached, as seen in *Figure 1.3.* (Kojima et al., 1999). However, the two ghrelin isoforms are not present in the same concentration *in vivo.* In rats, blood des-acyl ghrelin concentration is approximately twice that of acyl ghrelin (Hosoda et al., 2000).

Ghrelin and growth hormone releasing hormone (GHRH), which is secreted from the hypothalamus to stimulate GH release, have been found to act synergistically to increase serum GH in humans (Hataya et al., 2001). However, one study has shown that a single dose of ghrelin injected into rats had no significant effect on GH (Kamegai et al., 2000). This study also demonstrated an increase in

hypothalamic agouti-related protein (AGRP) mRNA expression when rats were injected with ghrelin and failed to demonstrate a significant increase in mRNA expression of NPY, POMC, GHRH or somatostatin. This indicates a potential lack of stimulus.

3.4. Feed intake and macronutrient control

In addition to stimulating GH secretion via binding to GHS-R, ghrelin has been implicated in feed intake regulation. Ghrelin stimulates the release of the potent feed intake stimulators NPY and AGRP. Infusion of ghrelin in rodents increases feed intake regardless of light phase (Nakazato et al., 2001). Ovine studies have demonstrated that ghrelin does follow a diurnal rhythm; however, different feeding regimens influence these patterns (Sugino et al., 2002a; Sugino et al., 2002b). Fasting has been shown to significantly increase ghrelin mRNA (Toshinai et al., 2001). Vagus signaling is at least partially responsible for ghrelin stimulation, as even rats lacking vagus nerves will not experience elevated ghrelin concentrations after food deprivation (Williams et al., 2003b).

Recent studies investigating ghrelin's orexigenic capabilities through utilization of different diet types have demonstrated results that reveal discrepancies resulting from the primary macronutrient content in the diet. Some studies suggest that a high-fat diet suppresses blood ghrelin concentrations longer than a carbohydrate-rich diet (Beck et al., 2002; Erdmann et al., 2003; Vallejo-Cremades et al., 2004), others have observed that protein has the least potent ghrelin suppression abilities in rats (Vallejo-Cremades et al., 2004) or that protein-rich diets

actually stimulate blood ghrelin concentrations in humans (Erdmann et al., 2003). Some studies have reported a stronger inhibition of mRNA expression and blood concentrations of ghrelin induced by high carbohydrate diets compared to fat-rich diets in both rats (Sanchez et al., 2004) and humans (Monteleone et al., 2003). Preferential utilization of carbohydrate stores rather than fat stores has also been observed in rats, where ghrelin injection increased body fat mass but did not change energy expenditure or feed intake (Tschop et al., 2000). These conflicting results warrant further investigation into the possible species differences in macronutrient control of ghrelin production.

In addition to the possibility that dietary macronutrient type may be a factor in ghrelin suppression, there is also speculation that ghrelin may be under pancreatic control and that insulin and glucagon may be key regulators of ghrelin expression and/or secretion. The presence of novel ε cells in the murine and rat pancreas that produce ghrelin have been documented (Ueno et al., 2005; Wierup et al., 2004). In humans, both oral or infused glucose was shown to suppress plasma ghrelin, though glucose infusion produced a more rapid decline in plasma ghrelin compared to ingestion of glucose (Shiiya et al., 2002). This relationship with glucose was also demonstrated *in vitro*. The addition of des-acyl ghrelin and glucagon to porcine hepatocytes *in vitro* further inhibited glucose output compared to the addition of glucagon alone (Gauna et al., 2005). This demonstrates not only a possible role for the des-acyl form of ghrelin, but also demonstrates a possible relationship between ghrelin and glucagon in the control of glucose output.

Insulin also appears to play a role in ghrelin secretion. Within 30 minutes of

administration, insulin induced both a significant and rapid decrease in plasma ghrelin (Saad et al., 2002). Insulin significantly increased ghrelin mRNA within 1 hour of injection and maintained a significant increase in mRNA at 2 hours post-insulin injection (Toshinai et al., 2001). Increased plasma glucose could result from meal ingestion or mobilization of body stores. This could explain apparent contradictory findings, as high glucose from meal consumption would theoretically be associated with low ghrelin while increased glucose due to carbohydrate mobilization would theoretically be associated with high ghrelin. Indeed, a recent study examined the effects of intragastric glucose infusion with open or inhibited flow through the pylorus and found that blood glucose was only significantly increased if the pylorus was open and plasma ghrelin was only significantly decreased under open flow conditions (Williams et al., 2003a).

3.5. Potential relationship with adrenocorticotrophin hormone

Recently, ghrelin has been found to inhibit the mRNA expression of StAR, *P450scc*, and 3β*HSD* (Tena-Sempere, 2005). This finding suggests that ghrelin also directly affects the production of cortisol in the adrenal cortex, as the inhibition of these enzymes would reduce the transformation of cholesterol to cortisol. Evidence of depressed ghrelin secretion as a result of cortisol regulation was also observed in adrenalectomized rats (Proulx et al., 2005). Transition dairy cows fed twice their recommended energy requirement have increased blood ACTH while a large negative energy balance also corresponds to an increase in ACTH (Beerda et al., 2004). This creates the opportunity for a differential correlation, as ghrelin would

theoretically be positively correlated with ACTH in underfed animals but negatively correlated with ACTH in overfed animals. To support this hypothesis, intravenous infusion of ghrelin into fasted adult human males has resulted in increased GH, ACTH and cortisol (Arvat et al., 2001). However, one study examining a 24-hour pattern of ghrelin and cortisol in humans observed no correlation between ghrelin and cortisol, as ghrelin surged prior to meal ingestion and was suppressed following meal consumption while cortisol followed a circadian rhythm (Purnell et al., 2003). This could indicate that ACTH provides more of a regulatory control of ghrelin than does cortisol. Human subjects have also been used in infusion studies examining differential treatment effects of acylated versus non-acylated ghrelin. Though des-acyl ghrelin was initially thought to be completely inactive, new roles for this isoform have been discovered. In terms of the stress response, the acylated form of ghrelin was observed to increase plasma ACTH, cortisol and glucose and decrease serum insulin, co-administration of acylated and non-acylated ghrelin continued to induce increased ACTH and cortisol but not significantly influence insulin or glucose (Broglio et al., 2004a).

4. Leptin

4.1. Introduction to leptin

Leptin is a product of the *ob* ("obese") gene, which is composed of a highly conserved sequence that has little variation across species. A two amino acid difference exists between bovine and ovine leptin, while 18 amino acids differ between human and bovine leptin (Blache et al., 2000).

Leptin is a primary satiety hormone and is secreted by white adipose tissue (WAT) to suppress feed intake. As body condition increases, circulating leptin also increases, and vice versa (Delavaud et al., 2002; Liefers et al., 2003). However, recent studies investigating leptin expression in gastrointestinal tissues in monogastrics and ruminants has identified a possible novel function of this secondary expression in the control of glucose uptake and metabolism. The current hypothesis is that WAT is the primary source of plasma leptin while luminal leptin is secreted by gastrointestinal tissue

4.2. Leptin and the neuropeptide Y pathway

Neuropeptide Y and leptin receptors are co-localized in the hypothalamic arcuate nucleus in the murine brainstem. Findings in the ovine brainstem have suggested this co-localization may not exist in all species. Regardless of a lack of co-localization, NPY and leptin receptors were observed to be closely related (Mercer et al., 1998). Such a close biological interaction between leptin and NPY insinuates a key role for leptin in feed regulation, as NPY exerts strong stimulation of feed intake while leptin counteracts the effects of NPY. Decreased leptin secretion results in an increase in NPY secretion (Friedman and Halaas, 1998). Leptin injection decreases NPY synapses and excitatory inputs to NPY cells (Pinto et al., 2004) and suppresses the stimulatory effects of NPY on GH secretion (Garcia et al., 2004). Also located in the hypothalamic arcuate nucleus and co-localized with NPY and leptin receptors, POMC is available for cleavage to produce α-melanocyte stimulating hormone (α-MSH), a satiety hormone, and AGRP, which is an

antagonist of melanocortin receptors and aids in the stimulation of feed intake. Cocaine- and amphetamine- regulated transcript (CART) is expressed in the hypothalamic arcuate nucleus, paraventricular nucleus and lateral hypothalamus and when secreted acts to inhibit feed intake (Ellacott et al., 2006).

4.3. Actions of leptin secreted from adipose tissue

Leptin secreted from adipose tissue into the blood has been hypothesized to be involved in feed intake suppression, as opposed to the luminal leptin secreted from gastrointestinal tissue. Decreased plasma leptin secretion results from underfeeding in ruminants; however, leptin has been observed to rebound to basal concentrations or higher upon refeeding (Chilliard et al., 2001; Delavaud et al., 2002). Infusion of canola oil into the abomasum of dairy cows for 18 hours did not significantly alter expression of adipose leptin mRNA (Chelikani et al., 2004), solidifying a role for WAT leptin in long term satiety signaling. In terms of plasma leptin, Holstein cattle have higher basal circulatory concentrations as compared to Charolais, along with increased plasma insulin and glucagon (Bellmann et al., 2004).

Plasma leptin potentially elicits its effects through several isoforms of the leptin receptor (Ob-R). To date, Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf isoforms have been documented. The Ob-Rb leptin receptor is currently understood to be the only long form of the receptor, having a long intracellular sequence allowing its involvement in intracellular signaling via the JAK/STAT pathway. The short isoforms Ob-R a, c, d, and f have truncated intracellular domains and are

present in higher concentrations than Ob-Rb in peripheral tissues and could be involved in leptin transport and appetite control (Ingvartsen and Boisclair, 2001). The Ob-Ra receptor isoform has been shown to stimulate the cytoplasmic tyrosine kinase Janus kinase-2 (Jak2), insulin receptor substrate-I (IRS-I), and the mitogen-activated protein kinase (MAPK) pathways. The Ob-Rb isoform is the most highly expressed leptin receptor in the brain and is the only Ob-R isoform that stimulates not only the Jak2, IRS-I and MAPK pathways, but the signal transducer and activator of transcription-3 (STAT3) can also be phosphorylated and activated via the Ob-Rb receptor (Bjorbaek et al., 2001; Sahu, 2004; Zabeau et al., 2003). To better depict the relationship between Ob-Rb and these pathways, *Figure 1.4.* illustrates the general steps that proceed with Ob-R activation. Because of these relationships, Ob-Rb has been the most investigated receptor isoform.

The use of an extracellular region probe has revealed hybridization of leptin receptors in the choroid plexus, the dorso medial hypothalamic nuclei, the ventro medial hypothalamic nuclei and the lateral hypothalamic nuclei, demonstrating a strong possibility that the hypothalamus is indeed a target site of leptin (Fei et al., 1997). Though there have been some differences in brainstem regions of Ob-R mRNA expression between murine and ovine samples, Ob-R mRNA expression has been observed in various regions of the dorsal vagal complex which enervates the gastrointestinal tract, the heart and the larynx (Mercer et al., 1998). These findings suggest that leptin receptors could be involved in nerve signaling to the gastrointestinal tract.

When subcutaneously injected with GH, serum leptin decreases in early- to

mid- gestation dairy cows, but not in non-pregnant dairy cows, suggesting differential regulation of leptin depending on other physiological needs beyond maintenance (Sauerwein et al., 2004). To exclude the possibility of external hormonal control, studies examining GH release in adenohypophyseal explants harvested from ovariectomized cows were performed (Zieba et al., 2003). Treatment of the explants with leptin resulted in an inverse dose-dependent response in GHRH mediated GH release in explants harvested from fed cows but an increase in GH release from adenohypophyseal explants obtained from fasted cows. As plasma leptin would theoretically be elevated in fed cows, addition of leptin would decrease GH secretion, and GH stimulation of liver gluconeogenesis would cease. Fasted cows would theoretically have low concentrations of leptin, and the addition of leptin to explants harvested from these cows would perhaps be over-ridden by hormones that stimulate feed intake, and hence may explain the increased GH.

There is some uncertainty as to whether insulin directly regulates WAT leptin or whether insulin acts indirectly via control of glucose levels, leaving glucose as the actual metabolite that influences leptin concentrations (Chelikani et al., 2003; Leury et al., 2003; Mueller et al., 1998). Feed consumption increases blood glucose concentrations, and insulin is secreted by pancreatic β cells to induce glucose uptake by tissues. Glucose is then stored as glycogen or glycerol (Squires, 2003). Leptin in turn reduces lipogenesis and increases lipolysis and fatty acid oxidation, providing acetyl-CoA for energy production in the TCA cycle. This complementary relationship results in a positive correlation between leptin and insulin. Mueller et al.

(1998) examined leptin regulation using an *in vitro* method where adipocytes were extracted from fat pads of anaesthetized rats. In this study, glucose activity and glycolysis were both inhibited experimentally. Leptin increased as a result, leading Mueller and colleagues to conclude that leptin was influenced by glucose fluctuations and was indirectly influenced by insulin. Chelikani et al. (2003) studied multiparous dairy cows in early and late lactation following jugular infusions of glucose, lipids or saline. They suggested that there could be a period of insulin-insensitivity in early lactation, but that there is a reliance on insulin to stimulate glucose mobilization and clearance in late-lactation. Administration of a hyperinsulinemic-euglycemic clamp to dairy cows during late pregnancy and in early lactation also demonstrated that insulin infusions increased plasma leptin concentrations by 75% in late pregnancy and 13% in early lactation (Leury et al., 2003). This insulin infusion study suggests that insulin concentration is directly correlated with leptin concentration, as glucose fluctuations were not significantly affected by insulin infusion when physiological state and insulin concentrations were integrated into the statistical equation. Insulin has been demonstrated to inhibit dexamethasone mediated leptin upregulation in cultured adipocytes after 36 hours of treatment (Considine et al., 1997). This suggests a direct role of insulin in leptin regulation. Examination of the leptin signaling pathway has also offered substantial evidence of direct insulin control. As previously stated, leptin exerts it effects through the Jak2, STAT3, IRS-1 and MAPK pathways. Insulin has, incidentally, been observed to stimulate phosphorylation of Jak2 and STAT3 in the rat liver (Carvalheira et al., 2003) and hypothalamus (Carvalheira et al., 2001). Insulin's

ability to activate the same pathways as the leptin receptor suggests a positive correlation between insulin and Ob-R.

4.4. Luminal leptin

Investigation into Ob-R expression has recently illuminated several novel functions of leptin. Leptin expression has been identified in the rat fundic chief cells (secretory granules) and endocrine cells and human fundic parietal cells (Cammisotto et al., 2006; Cammisotto et al., 2005; Sobhani et al., 2000). To further contribute to leptin expression in the ruminant gastrointestinal tract, leptin mRNA expression was observed in the rumen, abomasum and duodenum in 3-week-old Holstein bull calves being fed milk replacer, while mRNA expression persisted in the duodenum of 13-week-old and adult male Holsteins (Yonekura et al., 2002). Glucose absorption may play a significant role in this physiological difference, as ruminants convert glucose into volatile fatty acids for absorption in the rumen, while remaining glucose is absorbed in the small intestine. Young ruminants rely to a greater extent on glucose absorption in the small intestine while their rumen continues to develop.

The long-form leptin receptor has been identified in the basolateral membrane and parietal cells in the fundic epithelium of the human stomach, as well as in the human antral epithelium (Sobhani et al., 2000). Ob-Rb was also identified in chief cells in the rat gastric fundus, but was present only in the rough endoplasmic reticulum, golgi apparatus and immature secretory granules, but not in mature secretory granules (Cammisotto et al., 2006). Cammisotto et al. (2006) identified the

presence of the soluble leptin receptor isoform (potentially Ob-Re) in secretory granules of chief cells and endocrine cells and the presence of 2 proteases (furin and PC7) in secretory granules of chief cells, but not in endocrine cells. The long-isoform Ob-Rb could potentially undergo proteolysis, producing the short soluble isoform, which is bound to leptin in the gastric juice for transport and action on nutrient absorption in the small intestine. This is also supported by research that identified leptin receptors in the rat duodenum, jejunum and ileum brush border, lamina propria and basolateral membrane (Barrenetxe et al., 2002; Cammisotto et al., 2005; Lostao et al., 1998). Similarly in humans, the long form Ob-Rb was identified in the duodenal brush border plasma membrane (Barrenetxe et al., 2002).

The precise regulatory role of leptin in glucose absorption have recently been investigated, glucose inhibitors have been observed to have the same effect as increased leptin, leading to a significant decrease in glucose uptake in the gut (Lostao et al., 1998). This suggests that leptin influences transport of glucose across the intestinal wall. Upon addition of luminal leptin, glucose induced increase of sodium glucose transporter (SGLT-1) abundance in the brush border of the small intestine is inhibited (Ducroc et al., 2005). In relation to stress, dexamethasone was observed to significantly decrease glucose absorption in the rat jejunum compared to control rats; however, this study observed that stress significantly inhibited glucose transporter-2 (GLUT-2) activity as opposed to SGLT-1 activity (Shepherd et al., 2004). The biological difference between SGLT-1 and GLUT-2 is active versus passive transport, respectively. The possible causative differences between their regulation and function could relate to the amount of glucose in the small intestine,

or perhaps simply that leptin and glucocorticoids inhibit separate glucose transporters.

5. Stearoyl-CoA Desaturase

5.1. Introduction to stearoyl-CoA desaturase

Leptin's satiety signaling also affects the enzymes involved in metabolism of lipids and glucose. By decreasing lipogenesis, leptin also decreases the activity and mRNA expression of an enzyme known as stearoyl-CoA desaturase (SCD). Stearoyl-CoA desaturase is involved in the biosynthesis of monounsaturated fatty acids (MUFAs) from saturated fatty acids (SFAs) via desaturation at the Δ9 bond to yield a Δ9-cis double bond (Dobrzyn and Ntambi, 2004). Stearoyl-CoA (18:0) and palmitoyl-CoA (16:0) are the primary SFAs that are desaturated to yield oleoyl- (c-18:1n-9) and palmitoleoyl-CoA (c-16:1n-9), for their respective incorporation into triacylglycerol and cholesteryl esters (Heinemann and Ozols, 2003; Ntambi, 1999), as seen in *Figure 1.5.*.

Stearoyl-CoA desaturase is part of a series of components involved in Δ9 desaturation. First, Cytrochrome b5 reductase oxidizes either nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH), which provides the electrons necessary for the reduction of the cytochrome b5 heme. Stearoyl-CoA desaturase then uses an oxygen molecule as an electron acceptor for the hydrogens removed during the desaturation process (Vance and Vance, 2002). Because of SCD's involvement in desaturation and consequent triacylglycerol and cholesteryl ester formation, numerous tissues have been

observed to have SCD mRNA expression. However, there have been various forms of SCD discovered in rodents and humans, each with specific expression tissues. Stearoyl-CoA desaturase-1 mRNA is expressed in white and brown adipose tissue, liver, heart and kidney tissues, while SCD2 mRNA is expressed in the brain, kidney, spleen, lung, heart, β cells, and adipose, SCD3 mRNA is expressed in the skin and SCD4 mRNA is expressed in heart tissue (Ntambi et al., 2004). Although these SCD isoforms have not been identified in ruminants, eight single nucleotide polymorphisms in the bovine SCD gene have been discovered and do cause some differences in intramuscular fat monounsaturated fatty acid content (Taniguchi et al., 2004b). However, differences in levels of SCD mRNA expression have been identified in subcutaneous fat of female versus male cattle (Chung et al., 2000), which correlates with adiposity and serum leptin concentrations in females as compared to males.

Age differences also exist, as SCD mRNA expression peaks at approximately 12 months of age in cattle, at which time lipogenesis also increases (Martin et al., 1999). Cattle breed also influenced SCD mRNA expression as Holsteins had lower SCD mRNA expression than Japanese Black cattle (Taniguchi et al., 2004a) possibly because Holsteins have smaller fat reserves compared to Japanese Black cattle; therefore, less SCD activity for desaturaion of SFAs for TAG storage.

5.2. Stearoyl-CoA desaturase and diet composition

Stearoyl-CoA desaturase is responsible for the desaturation of saturated

fatty acids, yielding oleoyl- and palmitoyl-CoA. These MUFAs are then incorporated into TAG via diacylglycerol acyltransferase (DGAT). Triacylglycerol is a form of energy storage, and hence the amount of TAG deposition and mobilization is affected by diet and hunger/satiety signals (Kim and Ntambi, 1999; Miyazaki and Ntambi, 2003). Incidentally, oleoyl- and palmitoyl-CoA are also utilized by acyl-CoA cholesterol acyltransferase (ACAT) to synthesize cholesterol esters (Miyazaki and Ntambi, 2003). Because palmitate is the main fatty acid involved in conversion of cholesterol to cholesteryl esters and these cholesteryl ester pools can be converted to free cholesterol by HSL for cortisol synthesis, SCD would logically be in an inverse relationship with cortisol secretion.

Not surprisingly, diet and other feed intake related hormones have been observed to affect SCD expression and enzymatic activity. Low fat diets (4% fat, as compared to the high fat diet which contained 60% fat) increased hepatic SCD1 mRNA expression in mice (Kakuma et al., 2002), while insulin and fructose were observed to increase SCD gene transcription (Ntambi, 1995). The *asebia* mouse strain (ab^J/ab^J) has a mutation in the SCD1 gene, rendering the gene null. These mice have decreased fat deposition, decreased plasma leptin concentrations, but increased feed intake compared to control mice (Cohen et al., 2002). These findings demonstrate that leptin and SCD have a positive correlation, as leptin is secreted from WAT and SCD is involved in the formation of WAT. Increased SCD expression promotes TAG formation and fatty acid storage, increasing leptin secretion and initiating suppression of feed intake. Similarly, SCD1 enzymatic activity is increased in leptin null mice (ob/ob mice); however, feed intake is also observed to be

increased (Cohen et al., 2002). Because leptin cannot be secreted by WAT in this circumstance to signal feed suppression, *ob/ob* mice lacking leptin secretory capabilities continue to eat and accumulate fat. This also demonstrates that SCD is not involved as a satiety signal, as increased SCD1 activity did not initiate feed suppression.

Conclusion

Feed intake is by no means excluded from the list of biological activities affected by stress. This includes not only direct influence, but also indirect influence on pathways that affect glucose and fatty acid metabolism. The potential effect of ACTH on mRNA expression of leptin, ghrelin and SCD and ultimately their influence on feed intake is apparent. Recent research has shown that leptin and ghrelin have much more important roles regulating feed intake than previously thought. Though specific regulatory relationships have yet to be solidified, in light of the recent discoveries the scientific community has focused on elucidating these pathways and their biological roles. It is hypothesized that ghrelin mRNA expression could be detected in numerous ruminant tissues, and that acute stress will stimulate ghrelin mRNA expression. Acute stress is also hypothesized to depress leptin and SCD mRNA expression in adipose tissue due to their contribution to satiety signaling and fatty acid storage.

As little research to date has focused on ghrelin mRNA expression in ruminants, the first objective of the present study was to examine relative ghrelin mRNA expression in ruminant tissues. Acute stress elicits a rapid whole body

response, with specific short-term regulation different from that induced by chronic stress. Our second objective was to examine how simulation of acute stress response by means of ACTH injection alters relative levels of ghrelin, leptin, SCD mRNA expression in various tissues.

Increases intake	Decreases intake	
Agouti-related protein	α-melanocyte stimulating hormone	Insulin-like growth factor (I, II)
β- Endorphin	Anorectin	Leptin
Dynorphin	Amylin	Motilin
Galanin	Bombesin	Neurotensin
Ghrelin	Caerulin	Neuropeptide K
Growth hormone-releasing hormone	Cholecystokinin	Norepinephrine(β)
Neuropeptide Y	CART	Obestatin
Norepinephrine (α_2)	Corticotrophin-releasing hormone	Oxytocin
Melanin-concentrating hormone	Dopamine	Satietin
Opoids	Enterostatin	Serotonin
Orexin A and Orexin B	Estrogen	Somatostatin
Progesterone	Gastrin-releasing peptide	Substance P
Peptide YY	Glucagon	Thyrotrophin-releasing hormone
	Glucagon-like peptide-1	Vasopressin
	Insulin	Xenin

Table 1.1. Signals that stimulate or suppress feed intake (Ingvartsen and Andersen, 2000; Sahu, 2004).

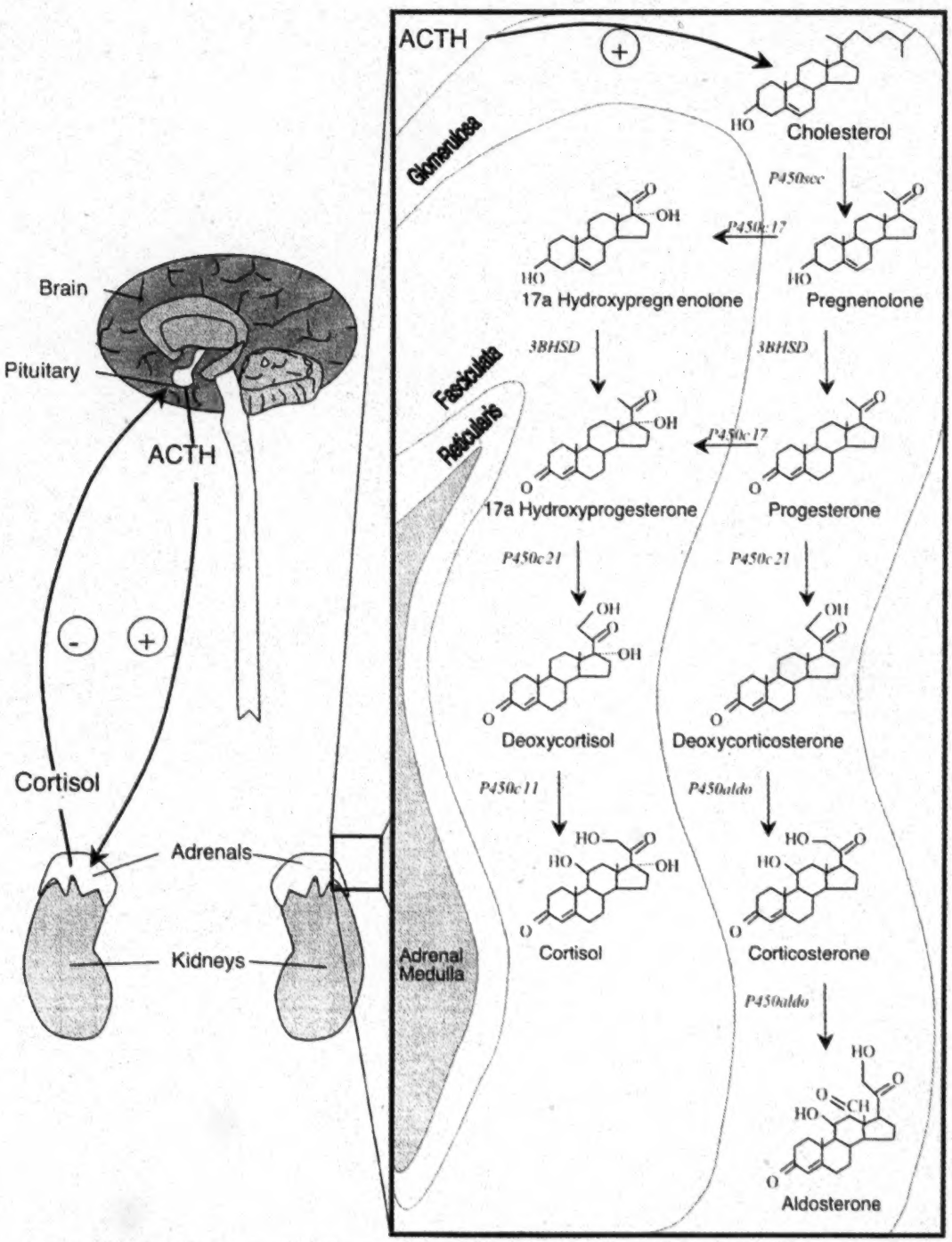

Figure 1.1. Schematic of feedback systems of adrenocorticotrophin hormone (ACTH) and cortisol between the anterior pituitary and adrenal cortex. Inset depicts structural stages of cortisol and aldosterone biosynthesis from cholesterol in the adrenal cortex regions (glomerulosa, fasciculata, reticularis) as stimulated by increased ACTH.

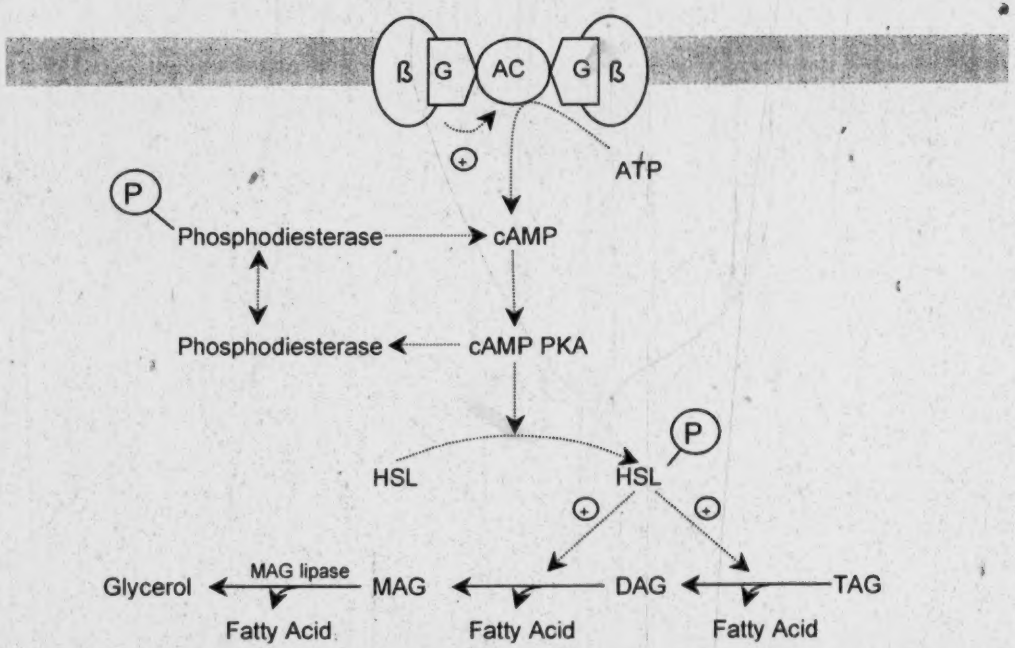

Figure 1.2. Stages of lipolysis. Catecholamines stimulate ß-adrenoreceptor (ß), which couples with the stimulatory G protein (G) to stimulate activation of adenylate cyclase (AC). Adenylate cyclase converts ATP to cAMP. cAMP then forms a complex with protein kinase A (PKA). PKA phosphorylates hormone sensitive lipase (HSL) to yield its active form. Active HSL is the key enzyme in conversion of triacylglycerol (TAG) and diacylglycerol (DAG) to yield DAG and monoacylglycerol (MAG), respectively. Cortisol also stimulates this pathway by promoting mRNA expression of the ß-adrenoreceptor and stimulatory G protein genes (Vance and Vance, 2002).

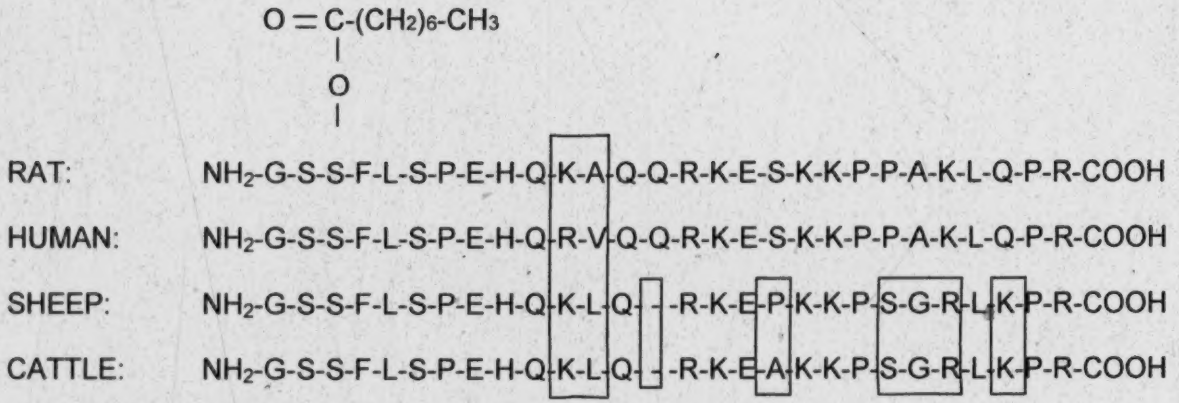

Figure 1.3. Structure of rat (Kojima et al., 1999), human (Ueno et al., 2005), sheep and cattle ghrelin (Bado et al., 1998; Kojima and Kangawa, 2005).

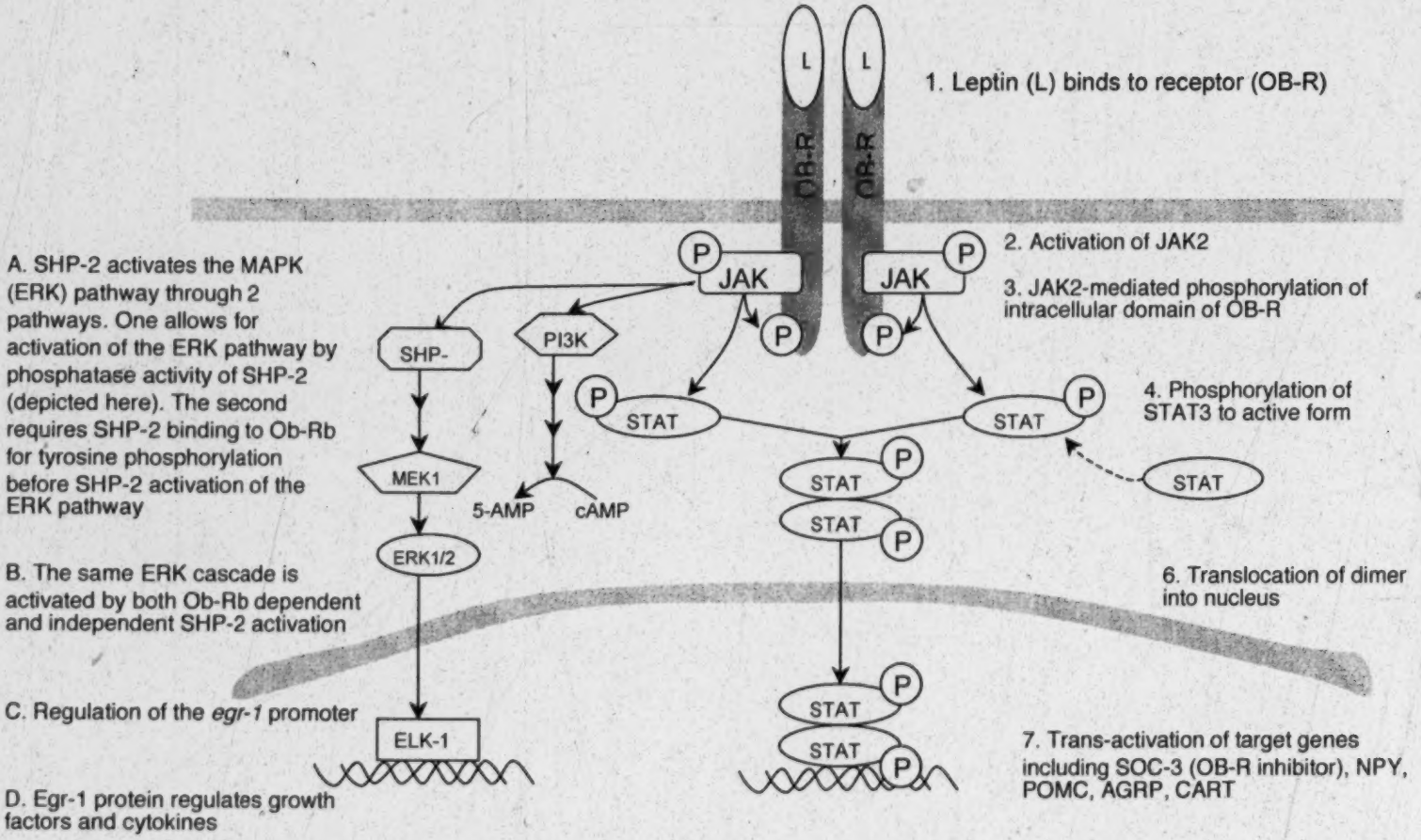
L
L
OB-R
OB-R
1. Leptin (L) binds to receptor (OB-R)
JAK
JAK
2. Activation of JAK2
3. JAK2-mediated phosphorylation of intracellular domain of OB-R
A. SHP-2 activates the MAPK (ERK) pathway through 2 pathways. One allows for activation of the ERK pathway by phosphatase activity of SHP-2 (depicted here). The second requires SHP-2 binding to Ob-Rb for tyrosine phosphorylation before SHP-2 activation of the ERK pathway
SHP-
PI3K
STAT
STAT
4. Phosphorylation of STAT3 to active form
MEK1
5-AMP
cAMP
STAT
STAT
STAT
ERK1/2
B. The same ERK cascade is activated by both Ob-Rb dependent and independent SHP-2 activation
6. Translocation of dimer into nucleus
C. Regulation of the egr-1 promoter
ELK-1
STAT
STAT
7. Trans-activation of target genes including SOC-3 (OB-R inhibitor), NPY, POMC, AGRP, CART
D. Egr-1 protein regulates growth factors and cytokines

Figure 1.4. Binding of leptin to its receptor (Ob-R) triggers the JAK/STAT pathway, as well as the PI3K and MAPK (ERK) pathway. Steps in the JAK/STAT pathway are depicted in Steps 1-7, while steps involved in the ERK pathway are outlined in Steps A-D. Diagram modified from Bjorbaek *et al.* (2001) and Sahu (2004).

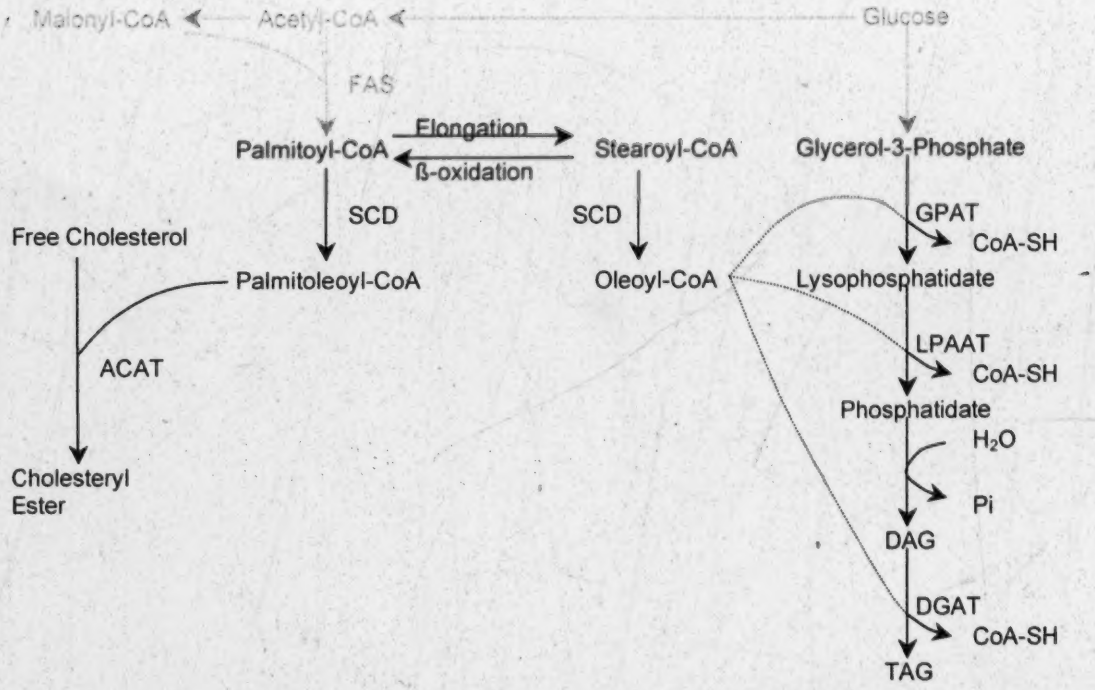

Figure 1.5. Desaturation of palmitoyl-CoA and stearoyl-CoA by stearoyl-CoA desaturase (SCD) yields monounsaturated fatty acids palmitoleoyl-CoA and Oleoyl-CoA, respectively. Each of these fatty acids has a particular role, as palmitoleoyl-CoA is used in conversion of cholesteryl esters to free cholesterol, while oleoyl-CoA can be bound to a glycerol backbone as a fatty acid storage mechanism.

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2. RELATIVE mRNA EXPRESSION OF GHRELIN, LEPTIN AND STEAROYL-CoA DESATURASE IN HOLSTEIN BULL CALVES INJECTED WITH ACTH

2.1. Introduction

Influence of acute and chronic stress in ruminants has been widely investigated for its implication in animal welfare (Friend, 1991). Little research to date has investigated how stress affects nutrient absorption and hormonal regulation of feed intake. Leptin and ghrelin are two hormones with counter-regulatory roles in feed intake. Monogastric studies identifying correlations between nutrient absorption and these hormones have suggested a more complex role than initially thought. Gastric and intestinal leptin is now thought to regulate glucose absorption in the small intestine (Ducroc et al., 2005; Lostao et al., 1998) and the ghrelin gene could potentially have regions that encode for contraindicative hormones, ghrelin and obestatin (Zhang et al., 2005). Ruminant studies have thus far demonstrated mRNA expression of leptin in the small intestine as opposed to the abomasum (Yonekura et al., 2002), while ghrelin mRNA expression is believed to be highest in the abomasum (Hayashida et al., 2001). Regulation of these hormones by adrenocorticotrophin hormone (ACTH) and cortisol appears likely, as cortisol itself affects nutrient absorption. The objectives of the current study were to examine fluctuations in relative mRNA expression of ghrelin, leptin and stearoyl-CoA desaturase (SCD) in tissues harvested from Holstein bull calves as a result of injection with ACTH and to determine sites of ghrelin expression in Holstein bull calf tissues.

In the following experiment it was hypothesized that plasma cortisol would significantly increase in calves injected with ACTH within 50 minutes post-injection.

Leptin mRNA expression was hypothesized to decrease in adipose tissue in the last time point sampled and to be positively correlated with SCD mRNA expression. Ghrelin mRNA expression was hypothesized to increase, with expression observed in numerous tissue types including the abomasum, small intestine, lung, heart and brain.

2.2. Materials and Methods

2.2.1. Animals

Twelve 5-month-old Holstein bull calves were used in this study. All animal treatments and sample collection was performed in 2002 by other investigators as part of a larger project and was originally intended for Microarray analysis. Tissue and plasma samples were stored at -80°C and -20°C, respectively, until work commenced on this study in 2005. Date of birth and birth weight of calves were recorded and are provided in *Table 2.1.*. Calves were weaned and fed a total mixed ration (TMR) according to University of Alberta Dairy Research Unit (DRU) protocol. Pooled feed intake of calves was recorded daily. Calves were moved from the University of Alberta DRU to the University of Alberta, Metabolic Unit on October 22nd, 2001 and were halter-trained prior to the experiment. All calves were housed separately from other animals housed at the Metabolic Unit and were euthanized individually in a room isolated from all other animals. Weights for calves euthanized on January 21st and 22nd, 2002 were determined on January 19th, 2002 and weights of calves euthanized January 28th, 2002 were determined on the day of euthanasia (listed in *Table 2.1.*). Final weights of calves (average 182.1 ± 20.9 kg) are also listed in *Table*

2.1..

2.2.2. Treatment

Each animal was injected and sacrificed separately. Animals were first stunned using a captive bolt pistol and were then killed by exsanguination in accordance with the University of Alberta Animal Care Committee Protocol. Adrenocorticotrophin hormone $(ACTH)_{(1\text{-}39)}$ from porcine pituitary was purchased from Sigma (Oakville, ON, Canada). Calves (3 calves/treatment group) were euthanized at 15 (AH15), 50 (AH50) or 100 (AH100) minutes following jugular injection with 0.56 IU/kg ACTH combined with saline to a total volume of 2 mL. The amount of ACTH administered was determined based on previous literature that demonstrated plasma ACTH and cortisol peaks significantly exceeding those observed in conjunction with average diurnal concentrations (Negrao et al., 2004). Control calves (SALINE, n=3) were sacrificed immediately following injection with 2mL of saline. Euthanasia was performed on four calves (one calf from each treatment group) on January 21st, January 22nd and January 28th. Calves were euthanized in the same treatment order on all trial days to minimize diurnal confounding factors. Time of injection and euthanasia are listed in *Table 2.2.*.

2.2.3. Sample collection

Tissues were harvested from calves, snap frozen in liquid nitrogen and stored at -80°C in sterile 50 mL polypropylene tubes until use for RNA isolation in 2005 (Fisher Scientific, Pittsburgh, PA, USA). *Table 2.3.* lists all tissue types collected from

each calf. Blood samples were collected at euthanasia. Whole blood samples were snap frozen in liquid nitrogen and stored at -80°C, while blood subsamples from calves 4041, 4043, 4051, 4047, 4052, 4042, 4053 and 4050 were also collected for plasma analysis and stored at -20°C until analysis was performed in 2006.

2.2.4. RNA isolation

RNA isolation was performed using TRIzol® (Invitrogen, Foster City, CA, USA). RNA from tissue types that had representation from at least 1 animal per treatment were used. Approximately 200-800 mg of frozen tissue was ground using sterilized chilled mortars and pestles. Frozen ground samples were homogenized in 1 mL TRIzol®/100 mg tissue using a homogenizer. Tissue weight was dependent upon tissue type. Samples were incubated for 5 minutes (30 minutes for fat samples) at room temperature. Chloroform was added to the samples at 0.2 mL/mL of TRIzol®. Samples were agitated and incubated at room temperature for 3 more minutes before centrifugation. The supernatant was removed and the remaining RNA pellet was washed twice with 1 mL of 75% ethanol/mL TRIzol®. The supernatant was discarded and the pellet was air dried before being resuspended in DEPC water and subsequently stored at -80°C. Absorbance was determined at 260nm and 280nm using the SpectroMax 190. RNA concentration was determined using the equation; A_{260} x dilution factor x 0.04. Two independent RNA subsamples were made from each original RNA sample to ensure homogeneity of sample and determine an accurate absorbance. All RNA was stored at -80°C until DNase treatment.

2.2.5. DNase treatment

DNase treatment was performed according to the manufacturer's conditions (Invitrogen, Foster City, CA, USA). Briefly, 0.5 μL DNase and 0.5 μL 10xBuffer were added to clean PCR tubes on ice. Autoclaved MilliQ water was added to 2.5 μg thawed RNA to produce a total volume of 5 μL. Samples were incubated at room temperature for 15 minutes and again for 10 minutes at 65°C after addition of 0.5 μL EDTA. Samples were then snap chilled on ice.

2.2.6. Reverse Transcription

In accordance with the product sheet (Invitrogen, Foster City, CA, USA), 0.25 μL Oligo DT (500 μg/ml) and 0.5 μL dNTP was added to chilled samples followed by pulse centrifugation. Samples were heated to 65°C for 5 minutes and were subsequently snap chilled on ice. Master mix containing 2 μL of First Stand Buffer, 0.25 μL RNase OUT and 1 μL DTT (0.1M) per sample was added. Samples were incubated at 42°C for 2 minutes prior to addition of 0.5 μL SuperScript II to each sample. Samples were then incubated at 42°C for 50 minutes and 70°C for 15 minutes. Portions of cDNA samples were diluted with autoclaved MilliQ water to achieve amplification within the standard curve. cDNA was stored as 1:1 and 1:80 stock at -20°C until thawed for Real-Time PCR.

2.2.7. Real-time polymerase chain reaction (RT-PCR)

Minor groove binding (MGB) primers and probes for P450c17α, ghrelin, leptin and SCD using a FAM dye layer for the probe were ordered from Assay by Design

(Applied Biosystems, Foster City, CA, USA). Cyclophilin MGB primers and probe designs using VIC dye layer for the probe were used in a previous study (Guercio, 2003). Messenger RNA sequences used for primer and probe design are given in *Figures 2.1.* to *2.5.*. Primer and probe sequences were the following; Cyclophilin Forward (5'-3'): CCCAGGCTAAGCCTTCCAA Cyclophilin Reverse (5'-3'): CCGATGTCCACGTCGAAGA Cyclophilin Probe (5'-3'): CCCAGTAACCCTCGAGTT; Ghrelin Forward (5'-3'): CCAGCATGGCCAGACGTT Ghrelin Reverse (5'-3'): GGGDDACTCACTCGTTAGC Ghrelin Probe (5'-3'): AAGCTGAAGAAACCC; Leptin Forward (5'-3'): CGCTGTGGACCCCTGTAT Leptin Reverse (5'-3'): CCTCCACGTAAGACAGATAGG Leptin Probe (5'-3'): CGATTCCTGTGGCTT; SCD Forward (5'-3'): GCCGAGAAGCTGGTGATGTT SCD Reverse (5'-3'): GCAGGATGAAGCACAACAACAG SCD Probe (5'-3'): CAGAGGAGGTACTACAAACC; P450c17α Forward (5'-3'): GTGGCCCCTACGCTGATC P450c17α Reverse (5'-3'): GTCTGTGCCTTTGTCAATGGTAAG and P450c17α Probe(5'-3'): ATTGACTCCAGCATTGG. In order to determine treatment effect on the housekeeping gene, one full set of tissue types were first analyzed per treatment and compared for fluctuations. All samples were analyzed in triplicate uniplex reactions. Total samples volumes were 25 μL per reaction, consisting of 12.5 μL Taqman Master Mix, 0.225 μL forward primer (100 μM), 0.225 μL reverse primer (100 μM), 0.05 μL probe (100 μM), 7.0 μL autoclaved MilliQ water, and 5.0 μL 1:80 dilution cDNA. Samples were amplified using the Prism 7700 Real-time PCR machine using the following cycle times;

i. 2 minutes at 50°C

ii. 10 minutes at 95°C

iii. 15 seconds at 95°C
iv. 1 minute at 60°C
} X 40 Cycles

Samples harvested from the adrenal cortex, pituitary, hypothalamus, pons, mesenteric fat, pericardial fat, subcutaneous fat, abomasum, duodenum, jejunum, ileum, aorta, endocardium, kidney, liver, lung, testis, epididymis and spleen were analyzed for cyclophilin, ghrelin, leptin and SCD mRNA expression. Adrenal cortex, pituitary, hypothalamus, pons, testis and epididymis were analyzed for P450c17α mRNA expression.

2.2.8. Plasma cortisol extraction

Plasma cortisol extraction and assay were performed using a Neogen Cortisol Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (Lexington, KY, USA). One mL ethyl ether was added to 100 μL plasma in a clean glass tube. Samples were vortexed for 30 seconds before pipetting the top organic phase into a clean glass tube. The solvent organic phase was evaporated under a nitrogen stream and the remaining residue was resuspended in 200 μL of diluted extraction buffer and vortexed. Samples were further diluted to a 1:4 dilution and vortexed.

2.2.9. Cortisol enzyme-linked immunosorbant assay (ELISA)

Standards and plate were prepared according to the Neogen protocol. Fifty μL

of standard or sample were added to an anti-Cortisol rabbit antibody precoated plate. Diluted enzyme conjugate was added to each well and the plate was covered, gently agitated and incubated at room temperature for 1 hour. Plate contents were then discarded and any residue was tapped out onto a lint-free towel. Each well was washed three times with 300 μL of diluted wash buffer and 150 μL of K-Blue substrate was added to each standard, sample and blank well. The contents of the plate were gently mixed and incubated at room temperature for 30 minutes. Following incubations the plate was agitated and subsequently read at 650 nm using the SpectroMax 190. All samples were tested in triplicate and outliers were discarded based on percentage discrepancy. Intra-assay CV was 6.25% (all samples were tested on the same plate).

2.2.10. Non-esterified fatty acid assay

A NEFA C kit purchased from Wako (Richmond, VA, USA) was used for determination of non-esterified fatty acids using a previously described protocol modification (Johnson and Peters, 1993), with the exception that colour reagent solutions were not diluted with phosphate buffer. Ten μL of standard or sample were added to a 96-well plate in triplicate followed by addition of 95 μL of Color Reagent A. The plate was gently agitated and incubated at room temperature for 30 minutes. One hundred and ninety five μL of Color Reagent B was added to each plate, mixed and left at room temperature to incubate for 30 minutes. The plate was again agitated and absorbance was read at 550 nm using the SpectroMax 190. Any outliers were discarded based on percentage discrepancy. Intra-assay CV was 7.72% (all samples

were tested on the same plate).

2.2.11. Statistical Analyses

Real-time results were analyzed using the Pfaffl Method (Pfaffl, 2001). Plate efficiency (E) for each plate was calculated using $E=10^{[-1/slope]}$. Slope was determined using serial dilutions (1:20, 1:80, 1:320, 1:1280, 1:5120) of abomasal tissue for ghrelin and cyclophilin, and subcutaneous fat for leptin and SCD. Average E for genes were as follows; Ghrelin: 2.016, Leptin: 2.288, SCD: 2.020, P450c17α: 2.010, and Cyclophilin: 1.939 used for Leptin, SCD and P450c17α while Cyclophilin: 1.962 was used for Ghrelin. All results were compared relative to pooled SALINE calves (termed CONTROL group) and were inputed into Equation 1:

$$\text{Ratio} = \frac{(E_{target})^{\Delta Ct_{target}(control-sample)}}{(E_{reference})^{\Delta Ct_{reference}(control-sample)}}$$

Treatment differences were analyzed in SAS using PROC MIXED lsmeans by tissue. To increase sample numbers per group, like tissues were pooled into the following categories:

1. Adrenal Cortex
2. Brain:
    a. Pituitary
    b. Hypothalamus
    c. Pons
3. Fat:
    a. Mesenteric
    b. Pericardial

    c. Subcutaneous
4. Gastrointestinal:
    a. Abomasum
    b. Duodenum
    c. Jejunum
    d. Ileum
5. Heart:
    a. Aorta
    b. Endocardium
6. Kidney
7. Liver
8. Lung
9. Reproductive:
    a. Testis
    b. Epididymis
10. Spleen

Tissue groups that had less than 2 samples per treatment were not used. The number of samples per tissue type per gene of interest are listed in *Table 2.6.*. Determination of any significant differences in plasma metabolite concentrations between SALINE and treatment groups was performed using SAS PROC MIXED lsmeans. For all statistical analyses, significance was stated at $p<0.05$.

2.3. Results

2.3.1. Real-time PCR

Ghrelin

Ghrelin mRNA expression was observed in all tissue types. Though comparison of expression within the same tissue type can be made using the Pfaffl Method, comparison of expression between different tissue types cannot be performed on this data set. *Table 2.4. (A to D)* depicts the number of successful sequence amplifications per tissue type.

Relative ghrelin mRNA expression within each treatment for each tissue type are listed in *Table 2.5.*. No significant fluctuations in mRNA expression were observed for any treatments or tissues.

Leptin

As illustrated in *Figure 2.6.*, a significant increase in leptin mRNA expression was observed in gastrointestinal tissue 50 minutes after ACTH injection ($p= 0.04$) compared to the CONTROL group. Gastrointestinal tissue was separated into abomasum and small intestine for leptin analysis. Leptin mRNA was significantly increased in the small intestine ($p=0.02$) compared to the CONTROL group. No significant differences between treatments were observed in brain, fat, lung, reproductive or splenic tissues ($p>0.1$, *Table 2.6.*). Leptin mRNA expression was undetectable in the liver, lung and heart tissue, and only a portion of the adrenal and kidney samples had successful cDNA amplification; therefore, these tissues were also excluded from analyses due to low sample representation.

SCD

Relative mRNA expression values of SCD are listed in *Table 2.7.*. Significant differences were observed in SCD mRNA expression and are depicted in *Figure 2.7.*. In the liver, SCD mRNA expression was observed to be significantly lower in AH15 ($p=0.02$) and AH50 ($p=0.03$) calves relative to the CONTROL group. No trends for mesenteric, pericardial and subcutaneous fat were not observed ($p>0.1$). Stearoyl-CoA desaturase mRNA expression was observed to be significantly higher in the spleen of AH100 calves relative to the CONTROL group ($p=0.03$).

P450c17α

There was a significant increase in P450c17α mRNA expression in the adrenal cortex in AH100 calves relative to the CONTROL group (p=0.03). Relative mRNA expression values for P450c17α for each tissue group and illustration of fluctuations in relative expression in adrenal cortex tissue are given in *Table 2.8.* and *Figure 2.8.*, respectively.

2.3.2. Plasma assays

Plasma Cortisol

Plasma cortisol concentrations peaked in calves euthanized 50 minutes post - injection relative to the SALINE group (p=0.05; *Figure 2.9.*). No significant difference was observed between the SALINE group and AH15 or AH100 calves, as shown in *Table 2.9.*.

Plasma Non-Esterified Fatty Acids (NEFA)

Although there was a numeric increase in plasma NEFA in AH15 calves compared to SALINE calves (listed in *Table 2.9.*), there was no statistically significant difference in plasma NEFA concentrations observed relative to SALINE calves for any of the treatment groups (*Figure 2.10.*).

2.4. Discussion

2.4.1. Plasma cortisol and P450c17α mRNA expression

Although catecholamine release occurs within seconds after stress stimuli, activation of the hypothalamic-pituitary-adrenal (HPA) axis takes several minutes or

hours. The release of adrenocorticotrophin hormone (ACTH) to stimulate cortisol biosynthesis was simulated in the present study, where ACTH was injected to induce a cortisol response. In this experiment, cortisol concentration was significantly increased in calves euthanized 50 minutes post injection. This surge in cortisol biosynthesis results from an acute ACTH response, where available cholesterol is used for cortisol production. Though initial cortisol synthesis can be achieved from enzymes already active within the adrenal cortex, abundance of the enzymes generally increase in order to maintain increased plasma cortisol circulation. Stimulation of adrenal P450c17α mRNA expression significantly increased at 100 minutes post ACTH injection, which was the last time point sampled. This was likely the result of cyclic adenosine monophosphate's (cAMP) transcriptional regulation of the CYP17 gene. In this regulatory pathway, there are two regions in which cAMP can regulate CYP17 (Lund et al., 1990; Zanger et al., 1991). These regions are specifically regulated through the protein kinase A (PKA) and protein kinase C (PKC) signal transduction pathways (Bakke and Lund, 1992; Lund et al., 1997). The amount of ACTH injected in the present study was an appropriate amount to elicit a cortisol response, as both plasma cortisol and P450c17α mRNA expression were increased.

2.4.2. Growth hormone and the neuropeptide Y signaling

Ghrelin binds to growth hormone secretagogue receptor (GHS-R) as an endogenous ligand to stimulate growth hormone (GH) secretion. Because of ghrelin's role as an endogenous ligand, it was surprizing that no significant fluctuation was observed in ghrelin mRNA expression. Numerous mechanisms related to ghrelin

secretion have been previously demonstrated to interact with ACTH and cortisol. Cortisol stimulates growth hormone releasing hormone (GHRH) secretion from the hypothalamus under acute stress, ultimately stimulating GH release from the anterior pituitary. Bolus injections of ACTH or hydrocortisone in humans increased serum GH concentrations (Raza et al., 1998).

Similarly, cortisol stimulates neuropeptide Y (NPY), which also increases GH secretion. While ghrelin is known as a GHS-R ligand involved in increasing GH secretion, stimulation through the NPY pathway would indicate more of a role for satiety signals. Stress has been previously observed to affect NPY mRNA expression, though the type of stress is an important factor in determining the repercussions on NPY expression. While chronic stress decreases NPY mRNA expression and protein levels (Kim et al., 2003; Spinazzi et al., 2005), acute stress induces an increase in NPY mRNA expression (Kas et al., 2005). Neuropeptide Y and ghrelin both stimulate feed intake and are positively correlated, as ghrelin exerts its orexigenic effects through the NPY pathway (Kojima and Kangawa, 2005). This correlation illuminates another possible indirect method of cortisol stimulation of ghrelin mRNA expression, and is also depicted in *Figure 2.11.*.

It is possible that with a larger sample number, more subtle fluctuations in ghrelin mRNA expression could be observed; however, with the sample number available in the present study only a numerical increase in relative ghrelin mRNA expression was observed in calves euthanized 50 minutes post-ACTH injection. It must also be noted that peak mRNA expression of ghrelin and leptin could occur anywhere between 16 and 99 minutes (between AH15 and AH100 treatment groups)

in the present study. Because animals were only tested once within this time interval (AH50), perhaps the upswing or downswing of regulation was caught for each and not the actual peak.

Similarly to GH, NPY has also been observed to increase leptin expression/secretion. Though it seems counterintuitive for GH and NPY to stimulate both ghrelin and leptin, which have opposing roles in feed intake regulation, NPY and glucocorticoids have both been observed to increase leptin mRNA in ruminant adipose tissue (Chilliard et al., 2001).

2.4.3. Leptin mRNA expression in gastrointestinal tissue

In the present study, leptin mRNA expression was observed to increase in the gastrointestinal tract, but remained unaffected in adipose tissues. Leptin mRNA expression was previously observed in the rat and human gastric regions (Bado et al., 1998; Sobhani et al., 2000). Leptin mRNA is upregulated in human cells and live rats as a result of glucocorticoid (dexamethasone) blockage of the STAT3 pathway (Ishida-Takahashi et al., 2004). Leptin mRNA has been observed in the rumen, abomasum and duodenum of Holstein bull calves at 3 weeks of age but only duodenal leptin mRNA persisted at 13 weeks of age (Yonekura et al., 2002). The present study is the first to demonstrate leptin mRNA expression in the bovine small intestine at approximately 5 months of age post-weaning. Leptin mRNA expression in the small intestine may still be of greater importance in the calf throughout ruminal development. A role of the small intestine in glucose absorbance would still be important, as glucose absorption does occur in the small intestine due to ruminal

escape of dietary starch.

Though plasma leptin is secreted by WAT to suppress feed intake, luminal leptin exerts its effects on glucose uptake via the gastrointestinal tract. Upon increased luminal leptin, abundance of the sodium-glucose co-transporter 1 (SGLT-1) in the brush border of the jejunum was not increased despite the presence of increased glucose (Ducroc et al., 2005; Lostao et al., 1998). This inhibition was observed for the mucosal side of the intestinal section but a time-delayed response was observed in the serosal portion of the jejunum (Ducroc et al., 2005).

After ruminal development, the majority of carbohydrates are absorbed in the form of volatile fatty acids from the rumen (Dukes et al., 1993). Though mRNA expression and protein abundance of glucose transporters along the small intestine were initially believed to be minimal in adult ruminants (Wood et al., 2000), studies have more recently proven that SGLT-1 and glucose transporter-2 (GLUT-2) mRNA expression are still present and that these transporters are responsible for absorption of glucose that has escaped absorption in the rumen in cattle fed both concentrate and forage diets (Rodriguez et al., 2004). Sodium glucose transporter-1 actively transports glucose from the lumen of the small intestine across the brush border apical membrane, with protein abundance increasing towards the distal end of the small intestine (Rodriguez et al., 2004). Facilitative glucose transporter GLUT-2 is also present along the small intestine for glucose absorption (Zhao et al., 1998) and has also been observed to be inhibited by chronic stress and dexamethasone (Shepherd et al., 2004). In our study, leptin mRNA expression increased in the small intestine as a result of ACTH injection. Subsequent plasma cortisol elevation may

indicate that increased luminal leptin was being produced and secreted, inducing an inhibition of SGLT-1 and/or GLUT-2 glucose transport from the intestinal lumen.

Cholecystokinin (CCK) could also play a role in leptin mRNA expression, as CCK is stimulated by gastrointestinal absorption of fatty acids and amino acids and previous relationship between these two hormones have been observed (Bado et al., 1998; Ellacott et al., 2006). Cholecystokinin is secreted by duodenal cells upon feeding to suppress feed intake, with amplified secretion in cattle fed a high fat diet (Choi and Palmquist, 1996; Choi et al., 2000; Suominen et al., 1998). Recently, CCK activity has been implicated in regions associated with the HPA axis, including the adrenals and pituitary (Malendowicz et al., 2003; Vermetten and Bremner, 2002), with a general agreement that CCK and ACTH secretion are positively correlated (Vermetten and Bremner, 2002). Leptin and CCK synergistically provoke a depression of feed intake in mice (Barrachina et al., 1997), and this interaction may create a possible mechanism for regulation in our study. Upon ACTH injection, CCK secretion could potentially increase, inducing an increase in leptin expression and/or secretion. In a previous study that depicted an inhibition of SGLT-1 in the presence of increased luminal leptin, blockage of the CCK-2 receptor abolished the inhibitory effect of leptin on the serosal side of the membrane (Ducroc et al., 2005). The ability to inhibit leptin response by inhibiting CCK demonstrates a strong interactive role of CCK and leptin control of glucose transport across the serosal membrane.

Looking at this response in a whole body context, a potential relationship can be forged. Upon ACTH injection, plasma cortisol immediately increases. This increase in plasma cortisol increases leptin mRNA expression in the small intestine

by blocking the STAT3 pathway. Once leptin mRNA in the small intestine is increased, luminal leptin can effectively block SGLT-1 absorption of glucose in the brush border (by PKC). If gut glucose and amino acid uptake is inhibited, CCK would be decreased. Also, since CCK and ghrelin are negatively correlated (Date et al., 2005), a decrease in CCK could allow for ghrelin mRNA expression and secretion to increase.

2.4.4. Lipolysis

Upon ACTH injection, increased glucocorticoid secretion from the adrenal zona fasciculata rapidly alters both carbohydrate and fat metabolism. However, glucocorticoids affect each organ differently and fat re-esterification is decreased and fat is mobilized from peripheral tissues and redistributed to centripetal organs (Dukes et al., 1993). It was anticipated that leptin mRNA expression would decrease in adipose tissue as a result of fatty acid mobilization and in turn plasma non-esterified fatty acid concentrations would increase. Neither one of these predictions were observed. The lack of leptin mRNA regulation in adipose tissue further supports the hypothesis that adipose leptin is more involved in long-term regulation of feed intake while luminal leptin is involved in more immediate carbohydrate regulation.

In the present study, no significant increase in the plasma non-esterified, or free, fatty acids was observed. This was unexpected, as cortisol is known to decrease re-esterification and ultimately increase fatty acid mobilization from lipid stores. One possible explanation could be that because 5-month-old calves were used in this study the amount of fatty acids mobilized from lipid stores would be naturally low

because these calves have not yet retained significant adipose reserves. Another possibility is that, even though there was a slight increase in fatty acids in the blood, other biological mechanisms were initiated in these calves to prevent the usage of their minimal energy reserves. One such mechanism used to prevent usage of adipose stores might be stearoyl-CoA desaturase.

Stearoyl-CoA desaturase (SCD) is involved in lipid storage, creating mono-unsaturated fatty acids to be stored as triacylglycerol (TAG). The liver and fat tissues harvested from calves euthanized 15 and 50 minutes post ACTH injection both displayed a decrease in SCD mRNA expression, suggesting an increase in fatty acid mobilization for energy usage. However, an increase in SCD mRNA expression was observed in the spleen of calves euthanized 100 minutes post-injection. There are several possible explanations for this finding. The first involves incorporation of oleate (18:1) in tissue around the spleen. Perfusion of the spleen with oleate demonstrated a higher affinity for its incorporation into phospholipids or NEFA in normal rat spleen. Inclusion of lipopolysaccharides resulted in a significant increase in esterification of oleate into TAG and cholesterol esters in the spleen (Hauton and Evans, 2002). Incorporation of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) into fatty acids close to lymph nodes has been previously observed to be higher in comparison to incorporation of saturated fatty acids (SFAs), and 18:1 levels have been observed to increase as samples were taken further away from the lymph nodes (Connor et al., 1996; Pond, 2005; Pond and Mattacks, 2003). Because SCD converts stearoyl-CoA (18:0) to oleoyl (18:1), SCD mRNA expression may be elevated as a result of shifts in 18:1 incorporation upon cortisol stimulation.

The level of SCD mRNA expression could simply compensate for lipid loss more rapidly after cortisol depletion compared to other tissues. As seen in *Figure 2.7.*, SCD mRNA expression numerically increased in the liver and spleen after decreasing immediately post ACTH injection. The splenic artery filters blood through the spleen before flowing through the hepatic artery to the liver. Perhaps the spleen first began to filter an increase in NEFA after cortisol injection, and splenic SCD mRNA expression was increased in preparation to restore adipose energy reserves and re-esterify these free fatty acids.

The ability for the spleen to store lipids has been previously proposed, where removal of the spleen in humans induced a significant increase in serum TAG concentrations (Schmidt et al., 1997). Upon cortisol stimulation, it is possible that splenic lipid pooling is induced. An increase in SCD for MUFA synthesis would facilitate this TAG formation. Insulin has also been observed to alter splenic lipogenesis in rats. A 3.7 fold increase in splenic lipogenesis was observed in the rat spleen upon glucose intake after fasting. When insulin secretion was inhibited, no increase in splenic lipogenesis was observed (Hulstijn et al., 1997). Perhaps elevated insulin in the case of acute stress could still possess dominant regulation to promote restoring fat reserves after an initial elevation in cortisol, providing interesting new interactions between insulin and cortisol in terms of energy allostasis.

2.5. Conclusion

In the present study, tissues harvested from 5-month-old Holstein bull calves were used to determine sites of ghrelin mRNA expression in young cattle. Using the

sensitive Real-time PCR technique, amplification of ghrelin mRNA expression was detected in all tissues. This provides an interesting insight, as recent monogastrics studies have outlined several novel function of ghrelin in the adrenals, lungs, liver, kidneys, testes and heart (Mori et al., 2000; Tena-Sempere, 2005; Tortorella et al., 2003). Presence of ghrelin mRNA expression in these tissues gives rise to possible local production and paracrine action of the ghrelin hormone.

The second objective of this study was to determine the effects of ACTH injection on relative mRNA expression of ghrelin, leptin and SCD mRNA expression. It was first demonstrated that the amount of ACTH injected did elicit a significant increase in plasma cortisol concentration and a subsequent significant increase in relative P450c17α mRNA expression in the adrenal cortex. As a result of ACTH injection, SCD mRNA expression was significantly decreased in the liver in calves euthanized 15 minutes and 50 minutes post-ACTH injection compared to SCD mRNA expression in the liver harvested from saline-injected calves. This coincides with the classical decrease in fatty acid re-esterification in response to increased plasma cortisol. However, there was a significant increase in relative splenic SCD mRNA expression in calves euthanized 100 minutes post-ACTH injection relative to calves injected with saline. This could indicate a role for the spleen in re-storing body fat stores, splenic SCD mRNA involvement in fat redistribution in response to cortisol, or could indicate a role for splenic SCD in terms of stress-stimulated immune function. A significant increase in relative leptin mRNA expression in the small intestine of calves euthanized 50 minutes post-ACTH injection compared to saline-injected calves also provides another novel function of luminal leptin, as these findings could implicate

luminal leptin in stress-induced inhibition of glucose uptake in the small intestine.

In conclusion, this study provides interesting new insight into possible sites of local ghrelin production for newly observed functions. This study has also highlighted possible novel effects of an acute stress on leptin and SCD mRNA expression in tissues that have thus far been understudied in terms of their stress-related role. However, because of the small sample number used in this study, further studies are required to verify these results and to determine whether ghrelin is produced from sites possessing ghrelin mRNA expression and confirm possible splenic SCD and luminal leptin involvement in the acute stress response.

Table 2.1. Calf information for Holstein bull calves. Birth date, birth weight, date of euthanasia, weight at euthanasia and estimated average daily gain based on pooled feed intake values and individual calf birth and euthanasia weights.

Calf Number	Date of Birth	Birth Weight (kg)	Date of Euthanasia	Euthanasia Weight (kg)	Estimated ADG (kg/day)
4041	26-Jul-2001	38.2	21-Jan-2002	227	1.05
4043	4-Aug-2001	37.3	22-Jan-2002	175	0.81
4048	8-Aug-2001	41.6	28-Jan-2002	204	0.94
4051	21-Aug-2001	49.2	21-Jan-2002	182	0.87
4047	7-Aug-2001	41.2	22-Jan-2002	153	0.69
4049	11-Aug-2001	44.7	28-Jan-2002	202	0.93
4052	23-Aug-2001	53.1	21-Jan-2002	179	0.83
4042	28-Jul-2001	43.5	22-Jan-2002	187	0.80
4055	30-Aug-2001	48.8	28-Jan-2002	161	0.74
4053	24-Aug-2001	41	21-Jan-2002	169	0.85
4050	15-Aug-2001	N/A	22-Jan-2002	183	N/A
4045	6-Aug-2001	31.1	28-Jan-2002	163	0.75

Table 2.2. Euthanasia Information for Holstein bull calves. Date of euthanasia, treatment administered, time of injection and time of euthanasia (24 hour clock). AH treatment groups were administered 0.56 IU/kg of ACTH as a jugular injection and euthanized at 15 (AH15), 50 (AH50) or 100 (AH100) minutes post-ACTH injection. SALINE calves were euthanized immediately following jugular saline injection.

Calf Number	Date of Euthanasia	Treatment	Time of Injection	Time of Euthanasia
4041	21-Jan-02	SALINE	925	925
4043	22-Jan-02	SALINE	917	917
4048	28-Jan-02	SALINE	929	930
4051	21-Jan-02	AH15	1047	1102
4047	22-Jan-02	AH15	1005	1020
4049	28-Jan-02	AH15	1010	1025
4052	21-Jan-02	AH50	1138	1233
4042	22-Jan-02	AH50	1037	1127
4055	28-Jan-02	AH50	1039	1129
4053	21-Jan-02	AH100	1207	1347
4050	22-Jan-02	AH100	1041	1221
4045	28-Jan-02	AH100	1042	1222

Table 2.3. Tissues harvested in 2002 from 5-month-old Holstein bull calves after captive bolt pistol and exsanguination. Tissues were harvested immediately, snap frozen in liquid nitrogen and subsequently stored at -80°C until use in 2005/2006. Tissues collected for calves are represented by an 'X', tissues not collected are represented by not available 'N/A'.

Treatment:	SALINE			AH15			AH50			AH100		
Calf Number	**4041**	**4043**	**4048**	**4051**	**4047**	**4049**	**4052**	**4042**	**4055**	**4053**	**4050**	**4045**
Adrenal Cortex	X	X	X	X	X	X	X	X	X	X	X	X
Hypothalamus	X	X	X	N/A	X	X	N/A	X	X	X	X	X
Pituitary	X	X	X	X	X	X	X	X	X	X	X	X
Pons	N/A	X	N/A	N/A	X	X	N/A	N/A	X	N/A	X	X
Lung	X	X	X	X	X	X	X	X	X	X	X	X
Liver	X	X	X	X	X	X	X	X	X	X	X	X
Spleen	X	X	X	X	X	X	X	X	X		X	X
Kidney	X	X	X	X	X	X	X	X	X	X	X	X
Testis	X	X	X	N/A	X	X	N/A	X	X	X	X	X
Epididymis	X	N/A	X	N/A	X	X	N/A	X	X	X	X	X
Mesenteric Lymph Node	N/A	X	X	N/A	N/A	X	N/A	N/A	X	N/A	N/A	X
Aorta	N/A	N/A	X	N/A	N/A	X	N/A	N/A	X	N/A	N/A	X
Gall bladder	N/A	N/A	X	N/A	N/A	X	N/A	N/A	X	N/A	N/A	X
Abomasum	N/A	X	X	X	X	X	X	X	X	X	X	X
Duodenum	N/A	X	X	X	X	X	X	X	X	X	X	X
Jejunum	X	X	X	X	X	X	X	X	X	X	X	X
Ileum	X	X	X	X	X	X	X	X	X	N/A	X	X
Endocardium	X	X	X	X	X	X	X	X	X	X	X	X
Peri.car. Fat	X	X	N/A	X	X	X	X	X	X	X	X	N/A
SQ fat	X	X	N/A	X	X	X	X	X	X	X	X	N/A
Mes. Fat	X	X	N/A	X	X	X	X	N/A	N/A	X	X	N/A

AATTCCGGAG GAAATTCATT CTGCTCCAAT GGTTCTTCGC GGACCTTGTG
ACTTTAGGGT TCGAGCTGCA CGGTGACCAC GCGGTGTTAC GAGGCGGTCA
TACACGCGTC CTAGGGCCAC AATGTCGCAT CCATCCCCCC AGGCTAAGCC
TTCCAACCCC AGTAACCCTC GAGTTTTCTT CGACGTGGAC ATCGGAGGGG
AGCGAGTTGG TCGAATTGTC TTAGAATTGT TTGCAGATAT TGTACCCAAA
ACTGCAGAAA ATTTTCGTGC ATTGTGTACA GGAGAAAAAG GCATTGGACC
CACCACTGGG AAACCTCTTC ATTTCAAAGG ATGTCCTTTC CATAGAATTA
TTAAGAAATT TATGATTCAG GGTGGAGACT TCTCAAATCA GAATGGGACA
GGTGGAGAAA GCATTTATGG TGAAAAATTT GAAGATGAAA ATTTCCATTA
TAAGCATGAC AAGGAGGGAT TGCTGAGCAT GGCCAACGCT GGCAGCAACA
CCAACGGCTC CCAGTTCTTC ATCACCACCG TTCCGACTCC GCATTTGGAT
GGGAAGCACG TAGTGTTTGG CCAAGTGATT AAAGGAATGG GTGTGGCAAA
GATTCTGGAA AATGTGGAGG TGAAAGGTGA AAAACCTGCC AAATTGTGCG
TTATTGCAGA ATGTGGAGAA CTGAAAGAAG GGGATGATTG GGGAATATTC
CCAAAGGATG GATCTGGTGA CAGTCACCCA GATTTCCCCG AGGACGCAGA
TGTGGATTTA AAAGATGTAG ATAAAATTTT ATTAATATCA GAAGACTTAA
AAAACATTGG AAATACGTTT TTCAAATCCC AGAACTGGGA GATGGCCATT
AAAAAATACA CAAAAGTTTT AAGGTATGTG GAAGGTTCCA GGGCTGCTGC
TGAGGACGCA GATGGAGCAA AGCTGCAGCC TGTCGCTTTG AGCTGCGTGC
TGAATATTGG CGCCTGTAAA CTGAAGATGT CTGATTGGCA GGGCGCAGTC
GATAGCTGTT TGGAGGCCCT TGAAATAGAC CCATCAAATA CCAAAGCACT
GTACCGTAGA GCCCAGGGAT GGCAAGGATT AAAAGAATAT GATCAAGCAT
TGGCTGATCT TAAGAAAGCT CAGGAAATAG CACCAGAAGA TAAAGCGATC
CAGGCAGAAT TGCTGAAAGT CAAGCAAAAG ATAAAGGCAC AGAAGGATAA
AGAGAAGGCA GCTTATGCAA AAATGTTTGC CTGATGAGG

Figure 2.1. Bovine cyclophilin mRNA sequence (GenBank Accession Number D14047). Single nucleotide polymorphisms or publication discrepancies are represented by an 'N', sequences used for primer (Forward and complementary Reverse) and probe (VIC dye layer) are underlined.

CATCTGCAGC CTGCTGCTGC TCAGCGTGCT CTGCATGGAC TTGGCCATGG
CGGGCTCCAG CTTTCTGAGC CCCGAACATC AGAAACTGCA GAGAAAGGAA
GCTAAGAAGC CATCAGGCAG ACTGAAGCCC CGGACCCTGG AAGGCCAGTT
TGACCCGGAG GTGGGGCTCA GTCCCTCCAG CATGGCCAGA CGTTGGGGAA
GTTTCTTCAG GACATCCTTT GGGAAGAAGC TGAAGAAACC CTGGCTAACG
AGTGAGTGGC CCTGGGACCA ACCACCTGTC CGTTCTCCCA CCCT

Figure 2.2. Bovine ghrelin mRNA sequence (GenBank Accession Numbers AF350329 and NM174067). Single nucleotide polymorphisms or publication discrepancies are represented by an 'N', sequences used for primer (Forward and complementary Reverse) and probe (FAM dye layer) are underlined.

ATGCGCTGTG GACCCCTGTA TCGATTCCTG TGGCTTTGGC CCTATCTGTC
TTACGTGGAG GCTGTGCCCA TCNGCAAGGT CCAGGATGAC ACCAAAACCC
TCATNAAGAC AATTGTCACC AGGATCAATG ACATCTCACA CACGCAGTCC
GTCTCCTCCA AACAGAGGGT CACTGGTTTG GACTTCATCC CTGGGCTCCA
CCCTCTCCTG AGTTTGTCCA AGATGGACCA GACATTGGCG ATCTACCAAC
AGATCCTCAC CAGTCTGCCT TCCAGAAATG TGGTCCAAAT ATCCAATGAC
CTGGAGAACC TCCGGGACCT TCTCCACCTG CTGGCCGCCT CCAAGAGCTG
CCCCTTGCCG CAGGTCAGGG CCCTGGAGAG CTTGGAGAGC TTGGGCGTTG
TCCTGGAAGC TTCCCTCTAC TCCACCGAGG TGGTGGCCCT GAGCCGGCTG
CAGGGGTCAC TACAGGACAT GTTGCGGCAG CTGGACCTCA GTCCCGGGTG
CTGA

Figure 2.3. Bovine leptin mRNA sequence (GenBank Accession Number BTU43943). Single nucleotide polymorphisms or publication discrepancies are represented by an 'N', sequences used for primer (Forward and complementary Reverse) and probe (FAM dye layer) are underlined.

TGCAGCGGAA GGTCCCGAGC GCAGCGCTGC GGATCCCCAC GCAAAAGCAG
GCTCAGGAAC TAGTCTACAC TCAGTTTGGA CTGCCCCGAA CTCCGCTCCG
CAGTCTCAGC CCCGAGAAAG TGATCCCAGT GTCTGAGAGC CCAGATGCCG
GCCCACNTGC TNCAAGAGGA GATCTCTAGC TCCTACACAA CCACCACCAC
CATCACAGCA CCTCCTTCCA GGGTCCTGCA GAATGGAGGG GGCAAATTGG
AGAAGACTCC CCTATACTTG GAAGAAGACA TCCGCCCTGA AATGAGAGAT
GACATCTATG ACCCAACTTA CCAGGATAAG GAGGGCCCAA AGCCCAAGCT
TGAGTATGTT TGGAGAAACA TCATCCTCAT GTCTCTGTTA CACTTGGGAG
CCCTATATGG GATCACATTG ATCCCCACCT GCAAGATATA CACCTATATC
TGGGTGTTAT TCTACTATCT GATGGGTGCC CTGGGCATCA CAGCAGGGGC
CCATCGCCTG TGGAGTCACC GAACCTACAA AGCTCGGCTG CCTCTGCGGG
TCTTCCTGAT CATTGGCAAC ACCATGGCGT TCCAGAATGA CGTTTTTGAA
TGGTCCCGAG ATCACCGTGC CCACCACAAG TTTTCAGAAA CGGATGCCGA
CCCCCACAAT TCCCGACGTG GCTTTTTCTT CTCTCACGTG GGTTGGCTGC
TTGTGCGCAA ACACCCAGCT GTCAAAGAAA AGGGTTCCAC GCTAAATTTA
TCCGACCTAA GAGCCGAGAA GCTGGTGATG TTCCAGAGGA GGTACTACAA
ACCTGGTGTC CTGTTGTTGT GCTTCATCCT GCCCACACTC GTGCCNTGGT
ATCTGTGGGA TGAAACGTTT CAAAACAGCC TGTTTTTTGC CACCTTATTC
CGTTANGCCC TTGGGCTCAA CGTCACCTGG CTGGTGAATA GTGCTGCCCA
TATGTATGGA TACCGCCCTT ATGACAAGAC CATCAACCCC CGAGAGAATA
TTCTGGTTTC CCTGGGAGCT GNGGGTGAGG GCTTCCACAA CTACCACCAC
ACCTTTCCTT ATGACTACTC AGCCAGTGAG TACCGCTGGC ACATCAACTT
TACCACGTTC TTCATTGATT GCATGGCTGC CATCGGTCTG GCTTATGACC
GGAAGAAAGT ATCCAAGGCT GCCATCTTGG CCAGGATAAN AAGAACTGGA
GAGGANAGCN ACAAGAGTNG CTGAATTTGT GGTCCCTTGG GTTCCTTTTC
CAAAAGCCAT CTGGGCAGAG GTTTAATGTT CTGTTTATTA ACTACTGAAT
AATGCTACCA GGATGCTAAA GATGACGTTA ACCCATTACA GTACAGTATT
CTTTAAAATT TTCTTTTTAA ATTGAAAGCC AACAACTCTG CCTTTATGAT
GCTAAGCTCA TGTTCTTATT TCTTCTCCTA TCTTCTTTCT CTTCTGTTCC
CATTATCCTT CCCTTTGTTT TGTCCCTGTC ACCTTCCTTT CTCCTTCTCC
TCATTGCCCC CCAGGCAAGC AGGTGGTCAG TCATTGGTGG GTTTCCAGCT
TCCAAAGCCT AGACAACCCT NCTGTAGTGC CAAACTAGTG GTCTTTGCCC
CGGCTGACCC TTTCCTTGAG CTGTCTCAGC TTTAAGGTGG ATGNCTCAAG
CTAGAGATAT GACAGAATCT TCTGGCAAGG GCCTTGATGA TCTTCAGCCC
AGACTTTTGC TAAATGAAAT GGAAAAATAA CTTTATTTTG GCACCAAACT
GAAAAAACAG GTCAATTGTC AGGGGAGAGA GTCAGCATGC ATGGTGTGAT
TGATAAATAG GATGAGTTGA AGTGGGAAAC AAGGCAGGAA GCTCCTGCTG
Sequence Continues…

Figure 2.4. Bovine stearoyl-CoA desaturase (SCD) mRNA sequence (GenBank Accession Numbers AY241932 and AF188710). Single nucleotide polymorphisms or publication discrepancies are represented by an 'N', sequences used for primer (Forward and complementary Reverse) and probe (FAM dye layer) are underlined.

ATGTGGCTGC TCCTGGCTGT CTTTCTGCTC ACCCTCGCCT ATTTATTTTG
GCCCAAGACC AAGCACTCTG GTGCCAAGTA CCCCAGGAGC CTCCCATCCC
TGCCCCTGGT GGGCAGCCTG CCGTTCCTCC CCAGACGTGG CCAGCAGCAC
AAGAACTTCT TCAAGCTGCA GGAAAAATAT GGCCCCATCT ATTCCTTTCG
TTTGGGTTCC AAGACGACTG TGATGATTGG ACACCACCAG TTGGCCAGGG
AGGTGCTTCT CAAGAAGGGC AAGGAATTCT CTGGGCGTCC CAAAGTGGCC
ACTCTAGACA TCCTGTCAGA CAACCAAAAG GGCATTGCCT TTGCCGACCA
TGGTGCCCAC TGGCAGCTGC ATCGGAAGCT GGCACTGAAT GCCTTTGCCC
TGTTCAAGGA TGGCAACCTG AAGTTAGAGA AGATCATTAA TCAGGAAGCC
AATGTGCTCT GTGATTTCCT GGCCACCCAG CATGGAGAGG CCATAGATCT
GTCCGAGCCT CTCTCTCTGG CGGTGACCAA CATAATCAGC TTTATCTGCT
TCAACTTCTC CTTCAAGAAT GAGGATCCTG CCCTGAAGGC CATACAAAAT
GTCAATGATG GCATCCTGGA GGTTCTGAGC AAGGAAGTTC TGTTAGACAT
ATTCCCTGTG CTGAAGATTT TCCCCAGCAA AGCCATGGAA AAGATGAAGG
GTTGTGTTCA AACGCGAAAA GAATTGCTGA ATGAAATCCT TGAAAAATGT
CAGGAGAACT TCAGCAGTGA TTCCATCACT AACTTGCTGC ACATACTGAT
CCAAGCCAAG GTGAATGCAG ACAATAACAA TGCTGGCCCA GACCAGGATT
CAAAGCTGCT TTCAAATAGA CACATGCTNG CTACNATAGG GGACATCTTC
GGGGCTGGTG TNGAGACCAC CACGTCTGTG ATAAAGTGGA TCGTGGCCTA
CCTGCTACAC CATCCTTCNT TGAAGAAGAG GATCCAGGAT GACATTGACC
AGATTATAGG TTTCAATCGC ACCCCAACCA TCAGTGACCG GAACCGCCTT
GTCCTGCTGG AGGCGACCAT CAGAGAAGTG CTCCGAATCC GGCCTGTGGC
CCCTACGCTG ATCCCCCACA AGGCTGTCAT TGACTCCAGC ATTGGCGACC
TTACCATTGA CAAAGGCACA GACGTTGTGG TCAACCTGTG GGCACTGCAT
CACAGTGAGA AGGAGTGGCA GCATCCCGAC CTGTTCATGC CCGAGCGCTT
CTTGGACCCC ACGGGGNCGC AACTCATCTC GCCATCNTTA AGCTACTTGC
CCTTTGGAGC AGGACCCCGC TCCTGCGTAG GTGAGATGCT AGCCCGCCAG
GAGCTCTTCC TCTTCATGTC CNGGCTGCTG CAGAGGTTCA ACCTGGAGAT
CCCGGATGAT GGGAAGCTAC CNTCTCTGGA GGGCCATGCC AGTCTCGTCT
TGCAGATCAA ACCTTTCAAG GTGAAGATCG AGGTGCGCCA GGCCTGGAAG
GAAGCCCAGG CTGAGGGTAG CACCNCATGA CTCCACCCTA TNTGACCCCC
ACCGCACAGA ATTAGAGGAG CTCCCCCACC CTCTCCCACC ATTCCTTCTT
CCTCCCCNCC CACTCTGCCT TCTTTCCCAG CCTGCAGCCC TGGCAGTGAT
GTNCATNAAA CAGTTTCTTT CTCCAAA

Figure 2.5. Bovine P450c17α mRNA sequence (GenBank Accession Numbers NM174304, BC110169 and M12547). Single nucleotide polymorphisms or publication discrepancies are represented by an 'N', sequences used for primer (Forward and complementary Reverse) and probe (FAM dye layer) are underlined.

Tables 2.4. A. Successful ghrelin mRNA sequence amplification information for tissue samples harvested from 5-month-old Holstein bull calves and analyzed using Real-time PCR. Potential sample number is given for each tissue category, as well as the number of tissue samples per treatment group that had successful amplification and the total percent successful amplification for tissues within each tissue category.

Tissue	Total Sample No.	Samples Amplified				% Amplified
		Saline	AH15	AH50	AH100	
Adrenal	12	3	2	1	3	75.0
Brain	28	7	4	5	8	85.7
Fat	33	5	6	4	6	63.6
Gastrointestinal	45	10	11	12	11	97.8
Abomasum	12	2	3	3	3	91.7
Heart	16	4	2	3	3	75.0
Kidney	12	2	1	3	3	75.0
Liver	12	3	1	2	1	58.3
Lung	12	3	3	3	3	100.0
Mesenteric LN	5	2	0	1	1	80.0
Reproductive	19	5	4	4	5	94.7
Spleen	11	3	3	3	2	100.0

Tables 2.4. B. Successful leptin mRNA sequence amplification information for tissue samples harvested from 5-month-old Holstein bull calves and analyzed using Real-time PCR. Potential sample number is given for each tissue category, as well as the number of tissue samples per treatment group that had successful amplification and the total percent successful amplification for tissues within each tissue category.

Tissue	Total Sample No.	Samples Amplified				% Amplified
		Saline	AH15	AH50	AH100	
Adrenal	12	3	0	1	0	33.3
Brain	28	5	1	4	2	42.9
Fat	33	6	8	6	6	78.8
Gastrointestinal	45	6	4	4	6	44.4
Small Intestine	33	5	2	2	3	36.4
Heart	16	1	1	1	1	25.0
Kidney	12	0	0	0	0	0.0
Liver	12	0	0	0	0	0.0
Lung	12	2	1	2	1	50.0
Mesenteric LN	5	1	1	1	1	80.0
Reproductive	19	4	2	2	4	63.2
Spleen	11	1	3	3	2	81.8

Tables 2.4. C. Successful stearoyl-CoA desaturase mRNA sequence amplification information for tissue samples harvested from 5-month-old Holstein bull calves and analyzed using Real-time PCR. Potential sample number is given for each tissue category, as well as the number of tissue samples per treatment group that had successful amplification and the total percent successful amplification for tissues within each tissue category.

Tissue	Total Sample No.	Samples Amplified				% Amplified
		Saline	AH15	AH50	AH100	
Adrenal	12	3	3	3	3	100.0
Brain	28	7	7	6	8	100.0
Fat	33	6	8	6	6	78.8
Gastrointestinal	45	10	12	12	11	100.0
Heart	16	4	4	4	3	93.8
Kidney	12	2	2	3	3	83.3
Liver	12	3	3	3	3	100.0
Lung	12	3	3	3	3	100.0
Mesenteric LN	5	2	1	1	1	100.0
Reproductive	19	5	4	4	5	94.7
Spleen	11	3	3	3	2	100.0

Tables 2.4. D. Successful P450c17α mRNA sequence amplification information for tissue samples harvested from 5-month-old Holstein bull calves and analyzed using Real-time PCR. Potential sample number is given for each tissue category, as well as the number of tissue samples per treatment group that had successful amplification and the total percent successful amplification for tissues within each tissue category.

Tissue	Total Sample No.	Samples Amplified				% Amplified
		Saline	AH15	AH50	AH100	
Adrenal	12	3	3	3	3	100.0
Brain	28	5	4	4	5	64.3
Reproductive	19	4	2	4	5	78.9

Table 2.5. Relative ghrelin mRNA expression and standard error (SE) values determined by Real-time PCR using the relative quantification method outlined by Pfaffl (2001). Tissues were collected from 5-month-old Holstein bull calves. Treatment calves were given jugular injection of 0.56 IU/kg of ACTH and euthanized 15 (AH15), 50 (AH50) or 100 (AH100) minutes post-ACTH injection. Relative mRNA values determined for calves euthanized immediately following a saline injection were pooled to create the CONTROL group.

Tissue	Treatment							
	CONTROL	SE	AH15	SE	AH50	SE	AH100	SE
Adrenal	1	1.44	0.62	1.02	0.20	1.44	0.46	0.83
Brain	1	0.77	0.82	0.67	0.49	0.60	1.06	0.47
Fat	1	74.69	0.87	52.81	168.58	64.68	12.75	52.81
Gastrointestinal	1	10.46	5.66	6.31	23.57*	6.04	1.22	6.31
Heart	1	4.97	0.14	4.97	8.48	4.05	0.21	4.05
Kidney	1	1.77	0.01	1.77	0.82	1.02	0.06	1.02
Liver	1	7.95	0.20	7.95	0.39	5.62	1.01	7.95
Lung	1	1.83	1.59	1.05	0.68	1.05	0.60	1.05
Mesenteric LN	1	6.80	0.30	6.80	0.76	6.80	0.24	6.80
Reproductive	1	1.63	3.87	1.16	0.88	1.16	0.62	1.03
Spleen	1	1.77	2.76	1.02	2.81	1.02	1.55	1.25

Table 2.6. Relative leptin mRNA expression and standard error (SE) values determined by Real-time PCR using the relative quantification method outlined by Pfaffl (2001). Tissues were collected from 5-month-old Holstein bull calves. Treatment calves were given jugular injection of 0.56 IU/kg of ACTH and euthanized 15 (AH15), 50 (AH50) or 100 (AH100) minutes post-ACTH injection. Relative mRNA values determined for calves euthanized immediately following a saline injection were pooled to create the CONTROL group. * denotes a significant difference ($p<0.05$) between the treatment value and the CONTROL value.

Tissue	Treatment							
	CONTROL	SE	AH15	SE	AH50	SE	AH100	SE
Adrenal	1	0.61	10.42*	0.61	0.69	0.61	N/A	N/A
Brain	1	2.81	0.22	4.86	5.93	2.43	0.52	3.44
Fat	1	22.29	1.46	13.65	35.43	15.76	5.85	15.76
Gastrointestinal	1	2.45	1.72	2.12	8.20*	2.12	3.15	1.73
Heart	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Kidney	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Liver	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lung	1	3.86	0.37	3.86	1.29	2.73	N/A	N/A
Mesenteric LN	1	14.64	148.45*	14.64	1.64	14.64	22.43	14.64
Reproductive	1	2.28	2.99	2.28	1.42	2.28	2.43	1.61
Spleen	1	94.46	5.58	54.54	112.67	54.54	116.61	66.79

Table 2.7. Relative SCD mRNA expression and standard error (SE) values determined by Real-time PCR using the relative quantification method outlined by Pfaffl (2001). Tissues were collected from 5-month-old Holstein bull calves. Treatment calves were given jugular injection of 0.56 IU/kg of ACTH and euthanized 15 (AH15), 50 (AH50) or 100 (AH100) minutes post-ACTH injection. Relative mRNA values determined for calves euthanized immediately following a saline injection were pooled to create the CONTROL group. * denotes a significant difference ($p<0.05$) between the treatment value and the CONTROL value.

Tissue	Treatment							
	Pooled	SE	AH15	SE	AH50	SE	AH100	SE
Adrenal	1	2.24	0.74	1.29	0.36	1.29	0.78	1.29
Brain	1	2.04	0.32	1.34	0.56	1.45	2.89	1.25
Fat	1	0.26	0.44	0.16	0.36	0.18	0.66	0.18
Gastrointestinal	1	0.63	1.72	0.37	1.53	0.37	1.26	0.38
Heart	1	0.82	1.24	0.58	0.62	0.58	0.35	0.67
Kidney	1	13.24	1.89	9.36	13.86	7.64	1.99	7.64
Liver	1	0.26	0.153*	0.15	0.22*	0.15	0.49	0.15
Lung	1	0.55	0.55	0.32	0.65	0.32	1.01	0.32
Mesenteric LN	1	0.99	10.57	0.99	1.23	0.99	3.79	0.99
Reproductive	1	0.52	0.59	0.37	0.17	0.37	0.76	0.33
Spleen	1	0.39	0.78	0.23	1.01	0.23	2.36*	0.28

Table 2.8. Relative P450c17α mRNA expression and standard error (SE) values determined by Real-time PCR using the relative quantification method outlined by Pfaffl (2001). Tissues were collected from 5-month-old Holstein bull calves. Treatment calves were given jugular injection of 0.56 IU/kg of ACTH and euthanized 15 (AH15), 50 (AH50) or 100 (AH100) minutes post-ACTH injection. Relative mRNA values determined for calves euthanized immediately following a saline injection were pooled to create the CONTROL group. * denotes a significant difference ($p<0.05$) between the treatment value and the CONTROL value.

Tissue	Treatment							
	Pooled	SE	AH15	SE	AH50	SE	AH100	SE
Adrenal	1	19.18	1.95	11.07	9.49	11.07	58.09*	11.07
Brain	1	210.34	1.79	148.73	324.33	148.73	64.08	133.03
Reproductive	1	1.12	0.86	1.12	0.41	0.79	2.07	0.71

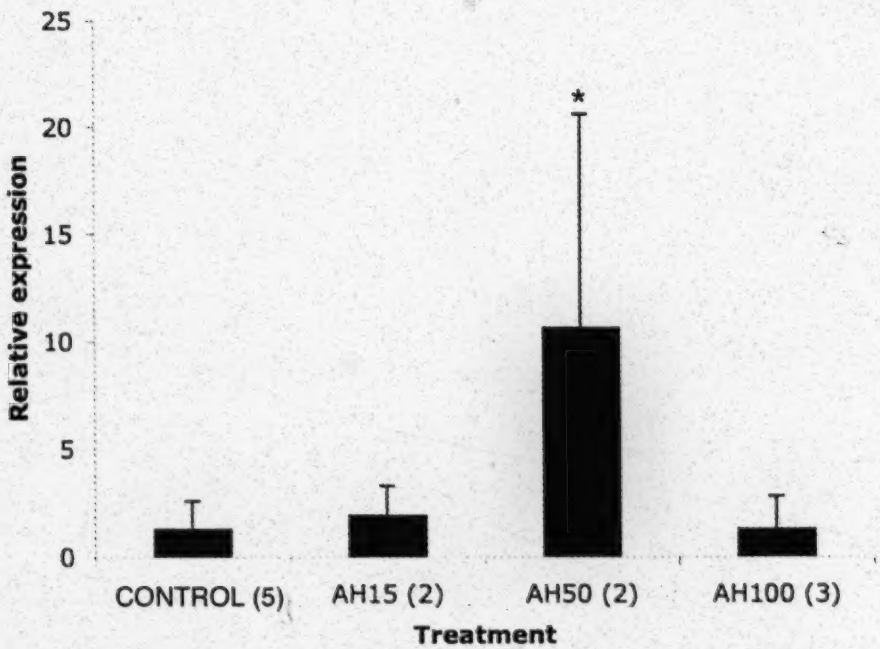

Figure 2.6. Relative leptin mRNA expression (mean ± SD) within the small intestine. Five-month-old Holstein bull calves were given a jugular injection of 0.56 IU/kg of ACTH and were euthanized 15 (AH15), 50 (AH50) or 100 (AH100) minutes post-ACTH injection, or were euthanized immediately following a jugular saline injection. Saline calves were then pooled to create the CONTROL group and all treatment groups were analyzed relative to the CONTROL group. The number of samples per treatment group are given in parentheses beside the treatment title. Relative leptin mRNA expression was higher (* $p<0.05$) in AH50 calves relative to the CONTROL group.

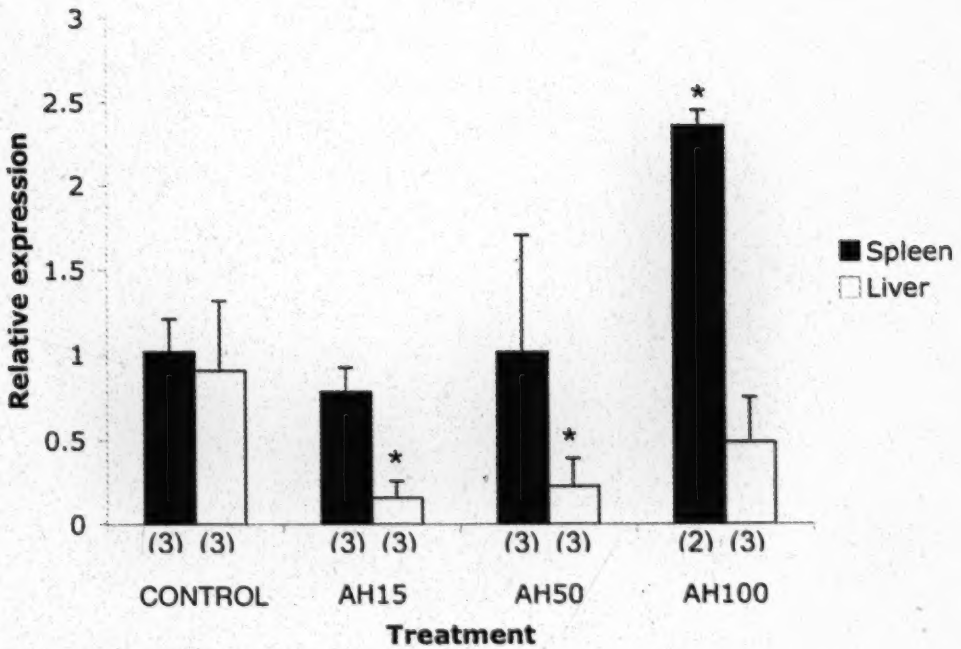

Figure 2.7. Relative stearoyl-CoA desaturase (SCD) mRNA expression (mean ± SD) within the liver and spleen. Five-month-old Holstein bull calves were given a jugular injection of 0.56 IU/kg of ACTH and were euthanized 15 (AH15), 50 (AH50) or 100 (AH100) minutes post-ACTH injection, or were euthanized immediately following a jugular saline injection. Saline calves were then pooled to create the CONTROL group and all treatment groups were analyzed relative to the CONTROL group. The number of samples per treatment group are given in parentheses beside the treatment title. Relative SCD mRNA expression was lower (* $p<0.05$) in liver tissue collected from AH15 and AH50 calves relative to the CONTROL group, while splenic SCD mRNA expression was significantly increased (* $p<0.05$) in AH50 calves relative to the CONTROL group.

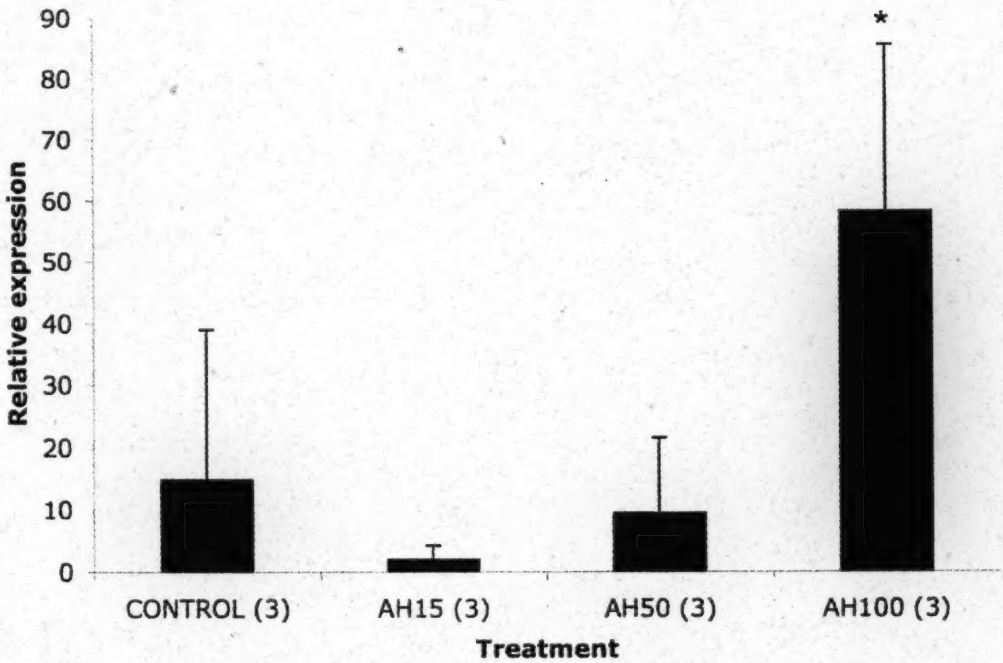

Figure 2.8. Relative P450c17α mRNA expression (mean ± SD) within the adrenal cortex. Five-month-old Holstein bull calves were given a jugular injection of 0.56 IU/kg of ACTH and were euthanized 15 (AH15), 50 (AH50) or 100 (AH100) minutes post-ACTH injection, or were euthanized immediately following a jugular saline injection. Saline calves were then pooled to create the CONTROL group and all treatment groups were analyzed relative to the CONTROL group. The number of samples per treatment group are given in parentheses beside the treatment title. Relative P450c17α mRNA expression was significantly increased (* $p<0.05$) in AH100 calves relative to the CONTROL group.

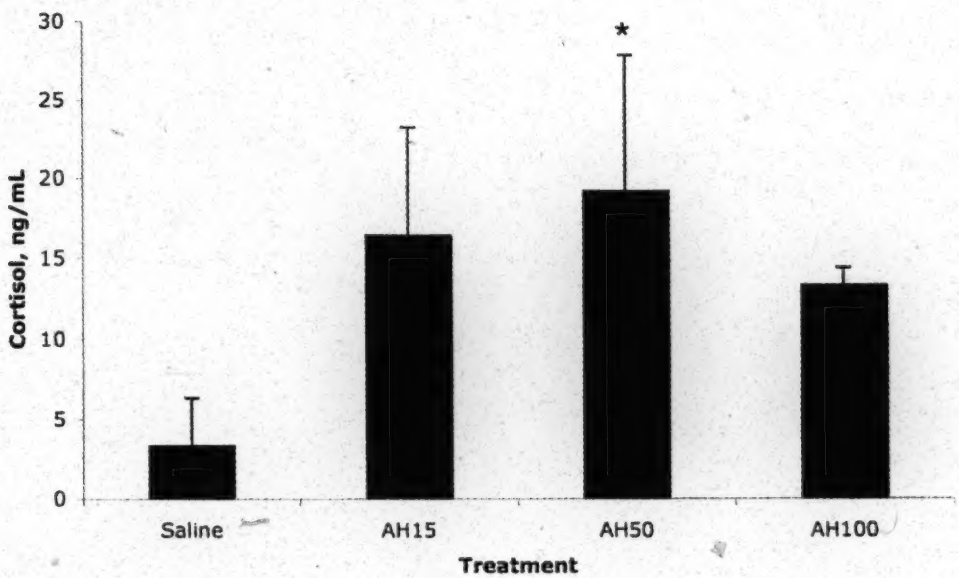

Figure 2.9. Plasma cortisol concentration (mean ± SD) determined from plasma collected from 5-month-old Holstein bull calves. Treatment calves were given a jugular injection of 0.56 IU/kg ACTH and were euthanized 15 (AH15), 50 (AH50), or 100 (AH100) minutes post-ACTH injection. Saline calves were euthanized immediately following a jugular injection of saline. Plasma cortisol was significantly higher (* $p<0.05$) in AH50 calves relative to the saline calves.

Table 2.9. Plasma cortisol (ng/mL) and plasma non-esterified fatty acid (mEq/L) concentrations observed for 5-month-old Holstein bull calves. Treatment groups were administered a jugular injection of 0.56 IU/kg ACTH and euthanized 15 (AH15), 50 (AH50) or 100 (AH100) minutes post-ACTH injection. The saline group was euthanized immediately following a jugular saline injection. Significance is noted as *$p<0.05$.

Metabolite	Treatment							
	Saline	SE	AH15	SE	AH50	SE	AH100	SE
Cortisol	3.38	5.66	16.46	5.66	19.24*	5.66	13.35	5.66
NEFA	0.14	0.04	0.17	0.04	0.12	0.04	0.10	0.04

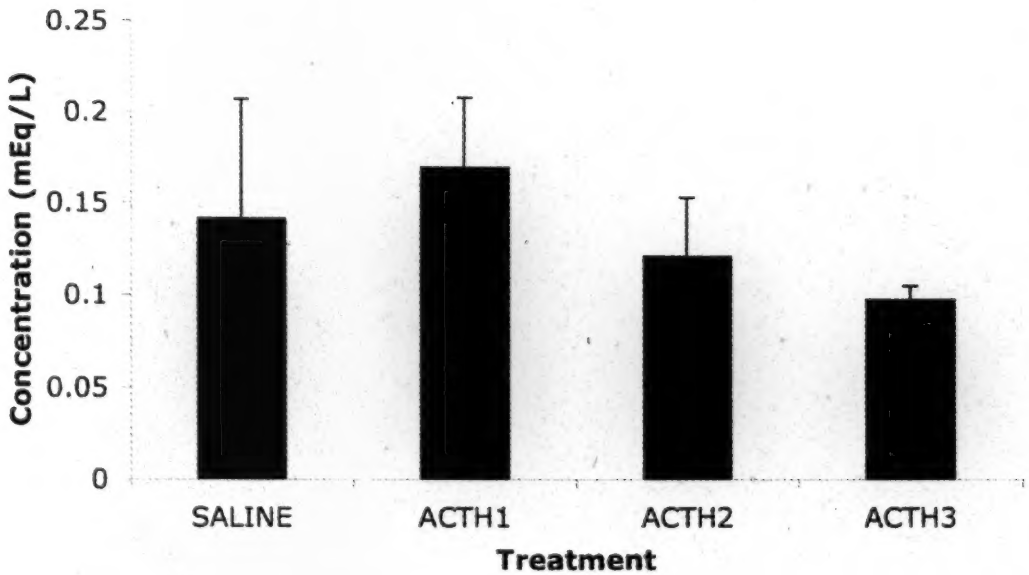

Figure 2.10. Plasma non-esterified fatty acid (NEFA) concentration (mean ± SD) determined from plasma collected from 5-month-old Holstein bull calves. Treatment calves were given a jugular injection of 0.56 IU/kg ACTH and were euthanized 15 (AH15), 50 (AH50), or 100 (AH100) minutes post-ACTH injection. Saline calves were euthanized immediately following a jugular injection of saline. There were no significant differences in NEFA concentration between any of the treatment groups and the saline calves.

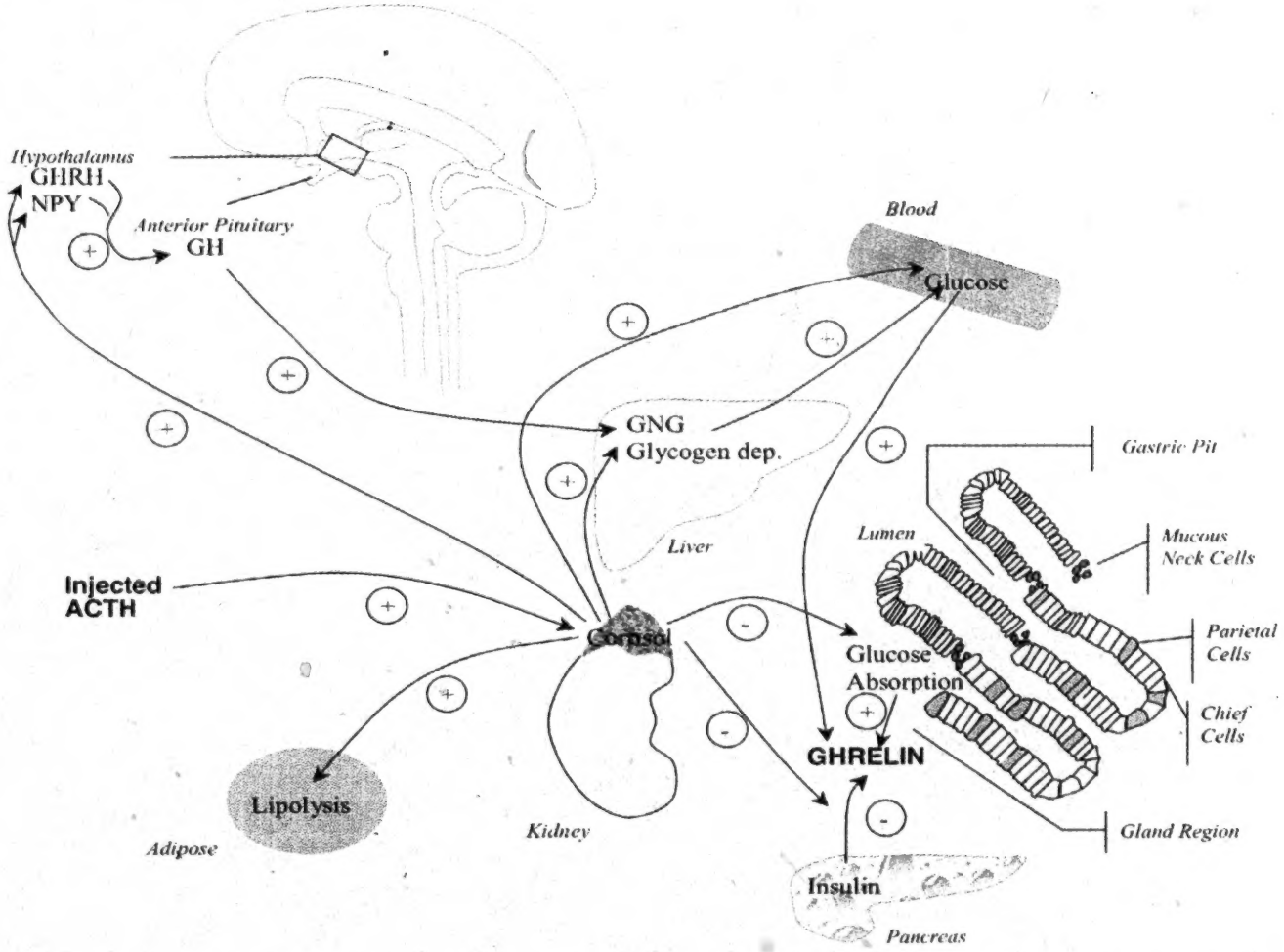

Figure 2.11. Illustration of possible pathways that ACTH elicits an increase in ghrelin mRNA expression in the abomasum.

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3. GENERAL DISCUSSION AND CONCLUSION

3.1. GENERAL DISCUSSION

Animal rearing, treatment and tissue collection for this study was performed by other investigators in 2002 as a part of another study that was designed for Microarray analysis and was not originally designed for the present experiment. Tissue and plasma analyses outlined in this dissertation did not occur until 2005/2006. During the period between collection and analyses, all tissue samples were stored at -80°C and all plasma samples were stored at -20°C.

It is necessary to note that any conclusions derived from this study must be interpreted with caution due to the small sample number; however, possible theories of biological mechanisms can be proposed to stimulate future research. The small sample number in the current study posed some difficulties when assessing fluctuations in mRNA expression, as a large variation in mRNA expression levels between animals was observed. With a larger sample size, it would have been possible to determine outliers and discard them from analysis. No mRNA expression data were discarded from the present analyses because a maximum of three calves per treatment were available and it was not possible to confidently determine outliers. Varying slaughter times of calves within specific treatments would also be recommended for future studies, as in this study calves within the same treatment were killed at approximately the same time of day. Diurnal fluctuations in hormones or enzymes could potentially play a factor. Study of mRNA expression but more importantly blood parameters would consequently be confounded.

Determination of pre-treatment concentrations of plasma cortisol for each

animal intended for this study would have been beneficial. Because of the high genetic variation of basal plasma cortisol concentrations between animals, it was possible that some animals had naturally high plasma cortisol even before any ACTH injection. Since no pre-treatment blood was available, it was impossible to provide any correlations between individual animals and consequent mRNA expression and plasma metabolite concentrations.

In this experiment, ACTH was injected into the blood, stimulating two stress pathways. Within seconds, catecholamine release from chromaffin cells are stimulated in the adrenal medulla. In the adrenal cortex, conversion of free cholesterol to cortisol is stimulated by ACTH within minutes (Dukes et al., 1993). Enzymes involved in cortisol biosynthesis are already present and active in the adrenal cortex to induce rapid cortisol synthesis and release into the bloodstream. In agreement with previous findings (Negrao et al., 2004; Zuber et al., 1985), plasma cortisol rapidly increased and peaked at 50 minutes post-ACTH injection, while P450c17α mRNA expression increased only by 100 minutes post-injection.

The differential action of glucocorticoids is evident in carbohydrate metabolism, where "anti-insulin effects" occur via increased glucose metabolism in the muscle and fat cells, while "insulin effects" stimulate liver glycogen deposition. An increase in lipid and cholesterol mobilization in the blood also occurs. Lipid stores are mobilized and redistributed in the body, with fat deposition in centripetal regions and fat loss in limbic regions (Dukes et al., 1993). Elevated cortisol decreases fatty acid re-esterification and TAG production in the fat and liver, acutely decreasing liver SCD mRNA expression within 15 minutes of ACTH injection. In the

present study, a significant rebound of splenic SCD mRNA expression occurred in calves euthanized 100 minutes post ACTH injection, compared to that of control calves euthanized after saline injection. Though research utilizing larger treatment numbers must be performed to confirm this, several viable explanations arise, including involvement of lipid pooling in the spleen or preferential usage of specific fatty acid types in the immunological response to acute stress.

Cortisol also inhibits glucose absorption by gastrointestinal tissue. Evidence of this is demonstrated by a significant increase in leptin mRNA expression in the small intestine at 50 minutes post-ACTH injection. There is strong evidence supporting the inhibitory role of luminal leptin on glucose transport across the intestinal brush border (Bado et al., 1998; Yonekura et al., 2002). Despite known stimulation of appetite via the NPY pathway under acute stress conditions, there was no significant increase in ghrelin mRNA expression. This was particularly surprizing considering ghrelin's positive correlation to both the NPY orexigenic pathway and its involvement in GH secretion (Kojima and Kangawa, 2005). This study provides insight into the possible relationships of ghrelin, leptin and SCD mRNA expression with ACTH and cortisol. However, further research is required, utilizing larger sample numbers and a tighter sampling timeline to confirm and clarify these findings.

3.2. FUTURE RESEARCH

1. *Examine the role of different diet types on luminal leptin and ghrelin mRNA expression in ruminants.*

It is unclear what ghrelin mRNA expression in the stomach reacts to, but it is clear that there is differential action (Erdmann et al., 2003; Monteleone et al., 2003; Sanchez et al., 2004; Vallejo-Cremades et al., 2004). In ruminants these effects could be unique because of volatile fatty acid production and absorption in the rumen. Cholecystokinin secretion has been previously observed to be preferentially secreted in response to high fat diets in cattle (Choi and Palmquist, 1996; Choi et al., 2000). It also seems logical that high carbohydrate ingestion would have a different stimulatory effect on luminal leptin secretion than would high fat, as luminal leptin is directly involved in glucose absorption while it is also influenced by cholecystokinin levels.

2. *Examine the relationship between luminal leptin and cholecystokinin.*

Though interaction between cholecystokinin and plasma leptin have been studied, the current theory is that plasma leptin is secreted from WAT and that luminal leptin is secreted from the stomach and small intestine. Dietary content is relevant when predicting the extent of cholecystokinin stimulation, as high fat diets have a greater stimulatory effect than other macronutrients (Choi and Palmquist, 1996; Choi et al., 2000). However, if luminal leptin secretion is affected more greatly by another macronutrient type then differential control, both synergistic or suppressive, could be observed.

3. *Determine relationship between cortisol secretion, age and leptin mRNA expression in the small intestine.*

Concentrations of plasma cortisol and leptin increase with age (Block et al., 2003; Negrao et al., 2004). However, how this affects luminal leptin is not fully understood. If calves are chronically stressed through out the developmental period, perhaps this induces leptin insensitivity. This could play a pivotal role in glucose and ultimately insulin levels in the body, or these factors could have a feedback role with luminal leptin.

4. *Determine regulatory roles of insulin on ghrelin mRNA expression.*

Contradictions exist in the literature as to the effects of both glucose and insulin on plasma ghrelin levels (Broglio et al., 2004; Williams et al., 2003; Yonekura et al., 2002). Both of these suggested relationships could, in fact, be true. The manner in which glucose and insulin are initially regulated could cause this discrepancy. Glucose ingestion could regulate ghrelin mRNA expression differently than increased blood glucose as a result of other factors, such as stress. Whether insulin has a direct role in ghrelin mRNA regulation or whether glucose is the primary source of control remains to be clarified.

5. *Identify the role of splenic SCD mRNA expression in lipid pooling and possible compensatory roles of the spleen in TAG storage.*

In the present study, it was interesting to note that even though SCD mRNA

expression decreased in liver, fat and spleen within 15 minutes post-ACTH injection, splenic SCD mRNA expression was observed to rapidly rebound significantly above basal expression levels. Reasoning behind this tissue specific compensation has yet to be studied. SCD mRNA expression in the spleen has received little attention to date, but the current investigation may indicate that TAG storage in the spleen could play an important role in the stress response.

6. *Further investigation of the relationship between plasma cortisol and SCD expression surrounding lymphatic tissues with specific emphasis on oleate formation.*

Determination of any correlations between SCD mRNA expression in splenic and lymphatic tissue and the stress response have yet to be determined. There is compelling evidence of differential usage of specific fatty acids surrounding and directly within lymphatic tissue. Mobilization of specific fatty acids in response to immune response has illuminated the fascinating possibility that perhaps stress also induces preferential utilization of unsaturated fatty acids in specific tissues. Involvement of specific fatty acids in the immunological component of the stress response may be the primary factor for the over-compensatory SCD mRNA expression in the spleen. However, further research must be conducted to confirm these findings and hypotheses.

3.3. CONCLUSIONS

This study provided a preliminary investigation into fluctuations in mRNA expression of key hormones related to feed intake, as well as a major enzyme involved in TAG storage in the body, as a result of ACTH injection. Increased cortisol biosynthesis was detected, both in terms of initial increase in plasma cortisol and increased mRNA expression of the CYP17 gene, which encodes for P450c17α, an enzyme involved in the cortisol biosynthetic pathway. Though regulation of SCD mRNA expression was observed, no significant fluctuation in plasma NEFA was observed, indicating that there was potentially a shift in unsaturation of fatty acids and not an increase in fatty acid mobilization. Also, no decrease in leptin mRNA expression was observed in adipose tissue. However, a significant increase in leptin mRNA expression in the small intestine was observed. Previous implication of luminal leptin in the inhibition of glucose transport across the intestinal brush border is suggested to be primarily responsible for the observed increase in expression. Ghrelin mRNA expression was also observed to increase in the gastrointestinal tract, indicating potential dual stimulation by hypothalamic control. However, the timeline for peak increased expression of these two opposing hormones could fall undetected between the first (15 minutes) or last (100 minutes) timepoints tested. Significantly increased mRNA expression of both leptin and ghrelin at 50 minutes post-ACTH injection could be before or beyond peak mRNA expression. Further investigation into acute stress control of both ghrelin and leptin mRNA expression will be needed to clarify these questions.

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Appendix-1: RNA isolation from tissues using TRIzol® Reagent

1. Wash mortars and pestles, wrap individually in tin foil and bake over 200° for 4 hours
2. After cooling, place mortars and pestles in -80°C freezer to chill
3. Put chilled mortar on dry ice and weigh empty 14 mL sterile polypropylene Falcon® tube (Becton Dickinson Labware, Le Pont De Claix, France)
4. Break loose a piece of frozen tissue into chilled mortar
5. Cover in liquid Nitrogen and grind with chilled pestle
6. Based on tissue type collect approximately 200 to 800 mg of ground tissue into pre-weighed 14 mL Falcon® tube
7. Store ground tissue in -80°C freezer until ready for RNA isolation
8. Add 1 mL TRIzol® Reagent (Invitrogen, Foster City, CA, USA) to ground tissue per 100 mg tissue
9. Homogenize the tissue for 30 seconds using a motorized homogenizer
10. Incubate the homogenate for 5 minutes (30 minutes for fat tissue) at room temperature
11. Add 0.2 mL chloroform to the Falcon® tubes per 1 mL TRIzol® Reagent added
12. Cap the Falcon® tubes and shake vigorously for 15 seconds
13. Incubate for 2 to 3 minutes at room temperature
14. Centrifuge the Falcon® tubes at 12,000xg for 15 minutes at 4°C

15. Pipette the top aqueous phase into a clean 14 mL Falcon® tube without disturbing the protein interface or top fat layer
16. Add 0.5 mL isopropyl alcohol per 1 mL TRIzol® Reagent
17. Incubate tubes for 1 hour at room temperature or overnight at -20°C for fat samples
18. Centrifuge at 12,000xg for 10 minutes at 4°C
19. Remove the supernatant without disturbing pellet
20. Wash the pellet with 1 mL 75% ethanol per 1 mL TRIzol® Reagent added
21. Centrifuge at 7,500xg for 5 minutes at 4°C
22. Aspirate the supernatant
23. Wash the pellet second time with 1 mL 75% ethanol per 1 mL TRIzol® Reagent added
24. Centrifuge at 7,500xg for 5 minutes at 4°C
25. Aspirate the supernatant and allow the pellet to air dry being careful not to overdry the pellet
26. Resuspend the pellet in autoclaved MilliQ water and transfer to 1.5 mL microfuge tubes (Fisher Scientific, Pittsburgh, PA, USA)
27. Determine absorbance at 260 nm and 280 nm
    a. Label clean 1.5 mL microfuge tubes
    b. Add 6 μL RNA to 554 μL Autoclaved MilliQ water in labeled microfuge tubes
    c. Pipette 250 μL diluted RNA sample in duplicate into wells on 96 well flat bottom UV plate (Corning Incorporate, Corning, NY, USA)

d. Read plate absorbance using SpectroMax 190 plate reader

28. Further dilute RNA if required and repeat absorbance reading

29. Store at -80°C until DNase treatment

Appendix-2: DNase treatment of RNA

Note: Invitrogen (Foster City, CA, USA) enzymes and buffers are used unless otherwise specified

1. Create Master mix of 0.5 μL amplification grade DNase and 0.5 μL 10xBuffer per sample
2. Add 1 μL DNase/Buffer Master mix to clean PCR tubes on ice
3. Add 2.5 μg RNA to tubes
4. Add Autoclaved MilliQ water to total 5 μL per tube
5. Pulse centrifuge
6. Incubate at room temperature for 15 minutes
7. Add 0.5 μL EDTA to each tube
8. Pulse centrifuge tubes to collect mixture
9. Incubate at 65°C for 10 minutes
10. Snap chill on ice

Appendix-3: Reverse transcription

Note: Invitrogen (Foster City, CA, USA) enzymes and buffers are used unless otherwise specified

1. Create Master mix containing 0.25 μL 500 μg/mL Oligo DT, and 0.5 μL dNTP per sample
2. Add 0.75 μL Master mix to tubes containing RNA
3. Pulse centrifuge to collect mixture
4. Heat samples to 65°C for 5 minutes and snap chill on ice
5. Create Master mix containing 2 μL First strand buffer, 0.25 μL RNase OUT, and 1 μL 0.1M DTT per sample
6. Add 3.25 μL of Master mix to tubes containing RNA
7. Incubate at 42°C for 2 minutes
8. Add 0.5 μL SuperScript II to samples
9. Pulse centrifuge
10. Incubate at 42°C for 50 minutes
11. Incubate at 70°C for 15 minutes
12. Pipette 2.5 μL cDNA into 197.5 μL Autoclaved MilliQ water in clean 0.2 mL PCR tube (Fisher Scientific, Pittsburgh, PA, USA) for 1:80 dilution
13. Store undiluted and 1:80 dilution cDNA at -20°C
14. For calf 43, take subsample of adrenal, abomasum and subcutaneous fat samples. Dilute 1:20, 1:80, 1:320, 1:1280, 1:5120 to create standards

Appendix-4: Real-time Polymerase Chain Reaction

Note: Invitrogen (Foster City, CA, USA) enzymes and buffers are used unless otherwise specified

1. Create Master mix containing 12.5 μL Taqman® Universal PCR Master Mix, 0.225 μL 100 μM Forward Primer, 0.225μL 100 μM Reverse Primer, 0.05 μL 100 μM Probe and 7.0 μL autoclaved MilliQ water per sample on ice
2. Pipette 20 μL Master mix into wells of 96 well-Optical Reaction Plate (Applied Biosystems, Foster City, CA, USA)
3. Add 5 μL of Standards (Abomasum Standards used for ghrelin and cyclophilin, subcutaneous fat for leptin and SCD, and adrenal for P450c17) or 1/80 sample cDNA to wells in triplicate
4. Cover plate with Thermal Seal sealing film (Axygen Scientific, USA)
5. Gently shake to mix the sample
6. Pulse centrifuge the plate to collect the mixture at the bottom of the wells
7. Place the plate in Prism 7700 Real-time equipment, cover with a compression pad and close lid
8. Create file specifying
    a. Standard concentrations
    b. Sample volume
    c. Probe dye layer (VIC for cyclophilin, FAM for P450c17, ghrelin, leptin, SCD)
    d. Cycles
        i. 2 minutes at 50°C
        ii. 10 minutes at 95°C

iii. 15 seconds at 95°C
iv. 1 minute at 60°C
} X 40 Cycles

e. Run Real-time Polymerase Chain Reaction

f. Upon completion of amplification, set threshold and background values, export data

Appendix-5: Cortisol Enzyme-linked immunosorbant assay (ELISA)

Cortisol Extraction

1. Dilute 'Wash buffer 10x' 10 fold with deionized water
2. Dilute 'Extraction buffer 5x' 5 fold with deionized water
3. Pipette 100 µL plasma into glass tube (Fisher Scientific, Pittsburgh, PA, USA)
4. Add 1 mL ethyl ether
5. Vortex for 30 seconds
6. Allow the phases to separate
7. Transfer the top organic phase into a clean glass tube
8. Evaporate the solvent under nitrogen gas
9. Resuspend the residue in 200 µL diluted extraction buffer
10. Add 40 µL of diluted cortisol to 120 µL diluted extraction buffer

Standards

1. Create the following stocks;

   A: Undiluted Standard

   B: 20 µL A + 980 µL EIA buffer

   C: 200 µL B + 1.8 mL EIA buffer

   D: 200 µL C + 1.8 mL EIA buffer

2. From stock, create the following standards;

   S_0 (0.00 ng/mL): EIA buffer as is

   S_1 (0.04 ng/mL): 800 µL EIA + 200 µL D

   S_2 (0.1 ng/mL): 500 µL EIA + 500 µL D

S_3 (0.2 ng/mL): D as is

S_4 (0.4 ng/mL): 800 µL EIA + 200 µL C

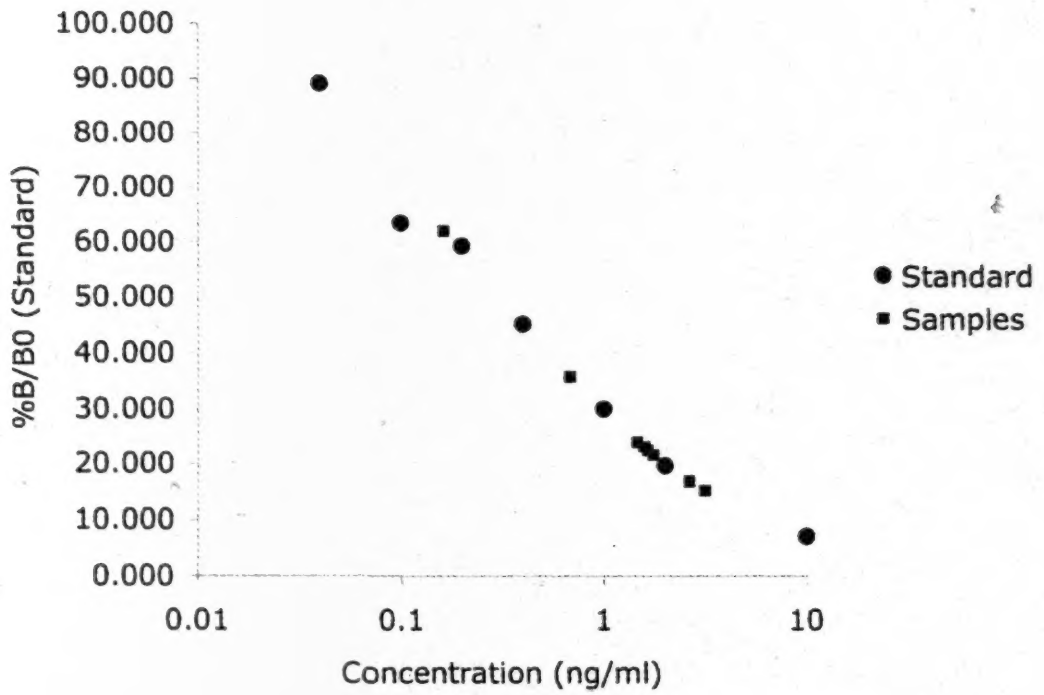
S_5 (1 ng/mL): 500 µL EIA + 500 µL C

S_6 (2 ng/mL): C as is

S_7 (10 ng/mL): 500 µL EIA + 500 µL B

Cortisol ELISA

1. In tube add 1 µL enzyme conjugate to 50 µL EIA per well to be used
2. Mix
3. Add 50 µL sample (Standard or unknown) to wells in a flat bottom UV plate (Corning Incorporated, Corning, NY, USA) in triplicate
4. Add 50 µL diluted enzyme conjugate to each well
5. Mix by shaking the plate gently
6. Cover and incubate the plate for 1 hour at room temperature
7. Dump out contents of the plate
8. Tap out contents on a clean lint free towel
9. Wash each well with 300 µL diluted wash buffer
10. Repeat wash 2 more times (3 washes total)
11. Add 150 µL K-Blue substrate to each well including the blank wells
12. Gently shake plate
13. Incubate at room temperature 30 minutes
14. Gently shake plate
15. Read at 650 nm



Cortisol 4-parameter Standard Curve determined using the Neogen Standard and samples collected from 5-month-old Holstein Bull calves.

Appendix-6: Non-esterified fatty acid enzyme immunoassay (EIA)

1. Create the following Standards;

   S_1 (1 mEq/L): undiluted Standard

   S_2 (0.5 mEq/L): 500 µL Phosphate buffer + 500 µL S_1

   S_3 (0.25 mEq/L): 500 µL Phosphate buffer + 500 µL S_2

   S_4 (0.125 mEq/L): 500 µL Phosphate buffer + 500 µL S_3

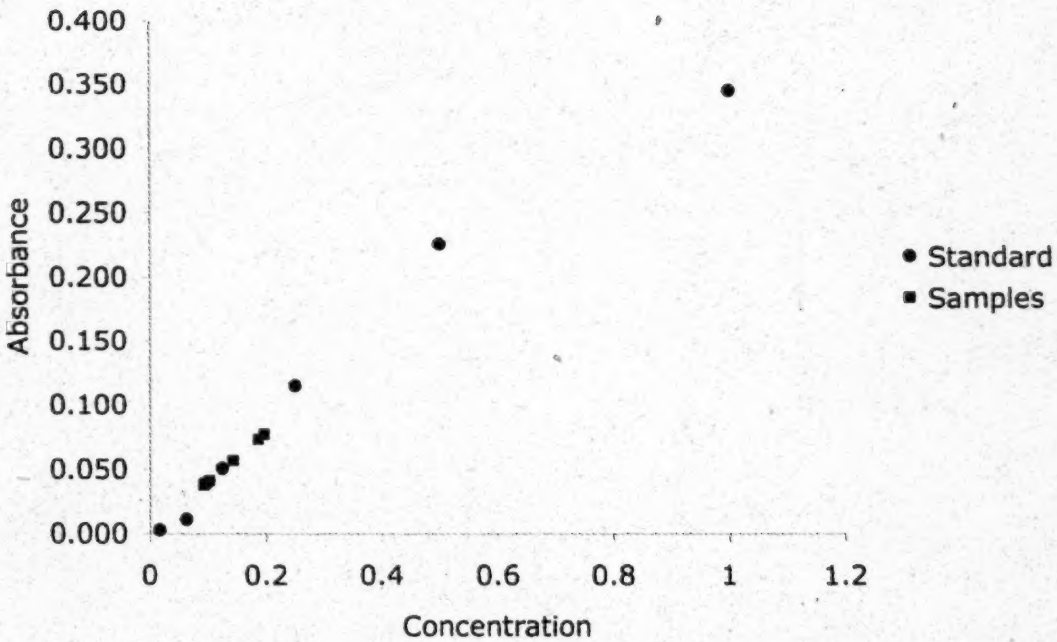
   S_5 (0.0625 mEq/L): 500 µL Phosphate buffer + 500 µL S_4

   S_6 (0.03125 mEq/L): 500 µL Phosphate buffer + 500 µL S_5

   S_7 (0.015625 mEq/L): 500 µL Phosphate buffer + 500 µL S_6

   S_8 (0.0078125 mEq/L): 500 µL Phosphate buffer + 500 µL S_7

2. Add 10 µL standard, sample or blank to wells in UV flat bottom 96-well plate (Corning Incorporated, Corning, NY, USA) in triplicate
3. Add 95 µL Color Reagent A
4. Mix gently
5. Incubate plate for 30 minutes at room temperature
6. Add 195 µL Color Reagent B
7. Mix gently
8. Incubate the plate 30 minutes at room temperature
9. Measure at 550nm



Plasma non-esterified fatty acid linear Standard Curve determined using the WAKO Standard and samples collected from 5-month-old Hosltein bull calves.